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EFFECT OF TEMPERATURE AND LIGHT ON DEVELOPMENT OF THE UREDIAL STAGE OF PUCCINIA GRAMINIS[1]

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INTRODUCTION

One of the important problems in the study of the epidemiology of stem rust, *Puccinia graminis* Pers., concerns the extent to which the uredial stage can persist through the winter under different conditions. The ability of this stage to survive obviously depends upon the ability of the urediospores or of the uredial mycelium in grains and grasses to withstand fluctuating temperatures and other conditions during the winter. It seems clear from the investigations of Stakman et al., as cited by Lambert (*12*),[3] that *P. graminis* does not commonly overwinter in the uredial stage north of Texas. As temperature, moisture, and light are likely to be among the most important physical factors involved, experiments were made to determine their effect.

HISTORICAL REVIEW

In the northern part of the United States the fate of the uredial stage of *Puccinia graminis* in the winter has been studied many years. In March 1905 Bolley and Pritchard (*4*) collected viable urediospores from leaves of quackgrass and wild barley frozen in the ice at Fargo, N. Dak. In 1906–07 Freeman and Johnson (*6*) were able to find viable urediospores on *Hordeum jubatum* L. and *Agropyron repens* (L.) Beauv. as late as April 15. In 1911 Hungerford (*10*) showed that urediospores of timothy stem rust, *P. graminis phlei-pratensis* (Eriks. and Henn.) Stak. and Piem., persisted through the winter in Wisconsin, while Stakman and Piemeisel (*24*) observed that they "survived the very severe winter of 1916–17 at St. Paul, Minn., very easily." It is evident, however, that *P. graminis phlei-pratensis* differs from other varieties of *P. graminis* in that its uredial stage can survive the winter in the northern United States more easily

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[3] Reference is made by number (italic) to Literature Cited, p. 879.

than can that of other varieties. Lambert (*12, p. 27*) has summarized some of the extensive studies of Stakman et al. made—

throughout the Mississippi Valley to determine the northern limits of overwintering of *P. graminis tritici* in the uredinial stage. * * * In general, they indicate that, north of Oklahoma, primary infection of cereals as a result of local overwintering in the uredinial stage, occurs so rarely that it is probably of no practical importance.

Hungerford (*11*) could not confirm the theory of Pritchard (*19, 20*) that the mycelium of *P. graminis* overwintered in seed wheat as mycelium and that when the seed germinated the mycelium grew by penetrating "various parts of the seedling, invading both the intercellular spaces and the cells."

It seems clear, therefore, that the uredial stage of most of the varieties of *Puccinia graminis* investigated does not commonly survive the winter in the northern United States. The question then arises whether urediospores and the uredial mycelium in perennial grasses and winter grains are killed by the cold or by alternate freezing and thawing. In an attempt to answer this question experiments were made to determine the resistance of urediospores and mycelium to low temperature as well as to fluctuating temperature. It is possible that the ability of urediospores to withstand extremely low temperature may depend somewhat upon the temperature and light conditions that prevailed prior to freezing. Experiments also were made, therefore, to determine the effect of temperature and light on the development of the uredial stage.

MATERIALS AND METHODS

Experiments on the effect of temperature and light were made in four large chambers kept at constant temperatures regulated by a large, automatically controlled refrigerating machine. The chambers were approximately 10 feet square and were lighted artificially by 1,000-watt incandescent lamps. To maintain uniform temperature and humidity, electric fans were kept running continuously. In addition to the four temperature chambers, an ice box cooled by a special refrigerating machine was used for extremely low temperatures. The greenhouse in which the plants were incubated in moist chambers was kept at about 20° C. by means of automatic heat controls.

Temperatures were recorded every 10 minutes by a Leeds-Northrop electrical recording machine and deviations from the desired temperature were corrected immediately, although the variation usually was not more than +1° C.

Several physiologic forms of *Puccinia graminis tritici* Eriks. and Henn., *P. graminis avenae* Eriks. and Henn., and *P. graminis secalis* Eriks. and Henn., and one collection each of *P. graminis phlei-pratensis* and *P. triticina* Eriks. were used. The histories of these forms are shown in table 1. The forms studied, except those of *P. graminis phlei-pratensis* and *P. triticina*, were cultured on some or all of the differentials of wheat, oats, and rye used in identifying the forms (*1, 5, 23*). The spore measurements were made under the 4-mm objective by means of a Zeiss ocular screw micrometer attached to a Spencer microscope.

Urediospores were hardened by placing plants heavily infected with the uredial stage in the 0° C. room for at least 10 days. This process was the same as that used by Harvey (*9*) for hardening higher plants before subjecting them to subzero temperatures.

TABLE 1.—*History of varieties and physiologic forms of Puccinia graminis and P. triticina used in studies of the effect of temperature and light on the uredial stage*

Species, variety, and form	Original host	Date collected	Locality in which collected	Collector
P. graminis tritici:				
Form 15	Wheat	May 12, 1923	Pusa, India	R. R. Sen.
Form 35	do	Sept. 2, 1926	St. Paul, Minn	E. Miller.
Form 36	do	Aug. 22, 1924	Morris, Minn	M. N. Levine.
P. graminis avenae:				
Form 5	Oats	Aug. 13, 1929	Morrisville, N. Y	Do.
Form 2	do	Aug. 17, 1925	Mankato, Minn	R. H. Bamberg, Lee Person.
P. graminis secalis:				
Form 7 (brown)	*Agropyron repens*	Aug. 9, 1927	St. Peter, Minn	C. L. Lefebvre et al.
Form 7 (orange)[1]	Rye	Mar. 3, 1929	Greenhouse, University Farm, St. Paul, Minn.	L. W. Melander.
P. graminis phlei-pratensis.	Timothy	Sept. 25, 1929	Scott County, Minn	Do.
P. triticina	Wheat	1930	St. Paul, Minn.[2]	

[1] Originated apparently as a mutation from the brown culture of *Puccinia graminis secalis* form 7; according to Cotter and Levine (*5*), there was no difference in the pathogenicity of the two strains.
[2] Contamination in greenhouse.

With few exceptions, the method of inoculation and incubation was uniform and similar to the one described by Cotter and Levine (*5*). Seedling plants first were sprayed with water and then inoculated by brushing them with rusted plants. The inoculated plants were placed in incubation chambers, again sprayed with water, kept at a temperature of 20° C. for 48 hours, and then placed under the conditions provided for a given experiment. All plants were watched closely for the first appearance of flecks and uredia. When the uredia had reached their maximum development, notes were taken on the number of plants inoculated and infected and on the type of infection produced.

Several experiments failed in the low light intensities because root rot killed the plants before rust pustules appeared. Thereafter all seed was treated with Ceresan [4] to delay damage from root rot until after uredia had developed and final notes had been taken.

RESULTS

EFFECT OF TEMPERATURE

In general, a temperature of 20° C. seems most favorable for the development of the uredial stage of *Puccinia graminis*, while subzero centigrade temperatures may kill urediospores, especially when moist, and temperatures just above zero may retard development by lengthening the incubation period and by preventing growth and sporulation. The effect of temperature may vary with varieties and physiologic forms. An account of the results of controlled experiments on these phases of the problem follows.

EFFECT OF CONSTANT SUBZERO CENTIGRADE TEMPERATURES ON VIABILITY OF UREDIOSPORES

The effect of outdoor conditions in winter on the viability of urediospores has been studied by various investigators (*3*, *4*, *6*, *17*, *18*, *20*),

[4] An organic mercury compound used in the treatment of seed for the prevention of numerous seed-borne diseases.

but very little is known concerning the effect of temperatures below 0° C. exclusive of other factors. Mehta (*16*) found that—

* * * after exposure to −6.5° to −10° C. for 24 hours, the urediniospores of this rust (*Puccinia graminis*) do not show even 10 percent germination and after four to seven days there is a total loss (only one or two spores germinating) of viability.

In the present work urediospores were exposed to temperatures of −29° to −40° C. constantly for various periods. The question arose whether hardening had any effect on the viability of urediospores. This is important because, in the Northern States, urediospores are exposed in the fall to temperatures just above 0° before the onset of cold winter weather. That fungi can be hardened has been demonstrated. Bartetzko (*2*), in 1909, showed that the minimum temperatures withstood by *Aspergillus niger* Van Tieg. could be lowered by first hardening, or exposing the fungus to a temperature just above freezing.

Numerous investigators (*4*, *5*, *6*) have observed that urediospores of *Puccinia triticina* live over the winter in the northern United States. In view of this fact, urediospores of this rust were frozen along with those of *P. graminis tritici*, *P. graminis avenae*, and *P. graminis phlei-pratensis*.

Spores of approximately the same age were dusted on dry glass slides in individual small pasteboard boxes. These were placed in an ice box maintained at temperatures below −29° C. Germination tests were made at the end of 1, 2, 3, 5, 10, 15, 20, 25, 30, 35, 40, and 45 days. Hardened spores were obtained from plants kept at 0° to 1° for at least 10 days after the uredia were mature, and nonhardened spores from plants inoculated at the same time but kept continuously in the greenhouse. After exposure to low temperatures, the spores to be tested were placed in a drop of distilled water on a glass slide and kept at 20° for 24 hours, after which counts were made.

That some urediospores of the *tritici* and *phlei-pratensis* varieties of *Puccinia graminis* can survive temperatures ranging from −29° to −40° C. for at least 45 days is shown in table 2. It appears that because of the sudden change in temperature more spores are killed during the first 24 hours than during any subsequent period. The viability of hardened spores of *P. graminis tritici* (table 2) was least affected by low temperature, and nonhardened spores of *P. graminis avenae* form 2 succumbed most readily, being unable to withstand the low temperature for more than 20 days in 2 out of 3 trials.

Hardened spores of *Puccinia graminis* withstood temperatures of −29° to −40° C. better than nonhardened spores (table 2 and fig. 1). While very few spores of *P. graminis avenae* survived, hardening seemed to enable some of them to survive prolonged exposure to −29° to −40°. There seemed to be little or no difference in ability of hardened and nonhardened urediospores of *P. triticina* to withstand low temperature.

Clearly, some urediospores of *Puccinia graminis*, when kept dry, can withstand considerable exposure to extremely low temperatures. This probably is true under natural conditions also, where long periods of continuous subzero centigrade temperature often prevail. The rather low temperatures of late fall are sufficient to harden urediospores and enable more of them to withstand constant subzero temperatures for long periods, but the question arises as to the effect on

the viability of urediospores of temperatures alternating above and below 0° C.

TABLE 2.—*Effect of hardening on viability of urediospores of Puccinia graminis and P. triticina at −29° to −40° C.*

Days at −29° to −40° C. (number)	Percent germination [1] of urediospores of—							
	P. graminis tritici form 15		*P. graminis avenae* form 2		*P. graminis phlei-pratensis*		*P. triticina*	
	Hardened	Nonhardened	Hardened	Nonhardened	Hardened	Nonhardened	Hardened	Nonhardened
0	41.3	55.2	17.6	33.5	45.7	28.5	17.1	40.1
1	6.1	3.0	6.9	2.0	5.7	1.6	10.2	2.7
2	6.4	6.6	1.9	1.3	3.3	.3	4.8	.5
3	10.0	5.2	.9	.3	3.4	.9	.0	3.4
5	24.1	7.0	.0	.0	3.9	.0	5.8	.0
10	7.2	.2	2.1	.2	8.5	.0	0.0	3.4
15	10.4	3.0	.9	1.1	8.0	.9	.9	.0
20	8.2	.6	.6	.0	5.3	.0	.0	.0
25	4.8	.0	.0	.0	.0	.0	2.9	3.0
30	9.4	.9	1.1	.0	3.2	.3	.0	1.7
35	8.1	.03	.0	.0	1.3	.2	.0	.0
40	1.2	1.1	.6	.0	1.9	.0	1.0	.0
45	2.5	.04	.0	.0	.9	.0	.0	.0

[1] Average of 3 trials in all cases except *P. triticina*, in which there were 1 and 2 trials, respectively.

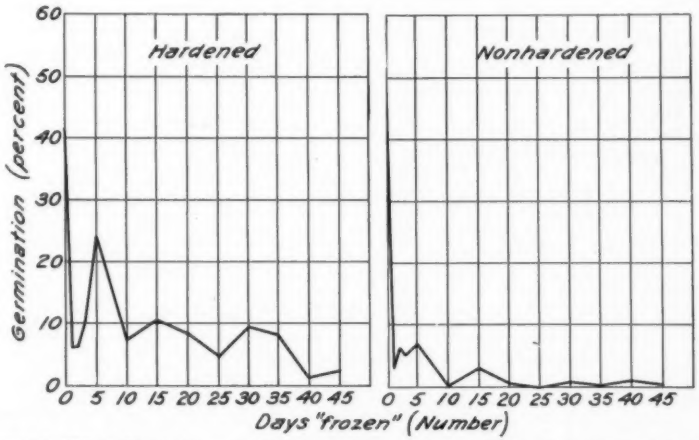

FIGURE 1.—Effect of hardening on resistance of urediospores of *Puccinia graminis tritici* form 15 to temperatures of −29° to −40° C.

EFFECT OF ALTERNATE FREEZING AND THAWING ON VIABILITY OF UREDIOSPORES

Stakman and Piemeisel (*24*) suggested that the reason urediospores of *Puccinia graminis tritici* do not survive the winter in Minnesota is that they are killed by "alternate freezing and thawing" in the spring. Lambert (*12*) also intimated that the reason urediospores survived the winter of 1923–24 at San Antonio and not at Dallas, Tex., was that alternate freezing and thawing occurred only 4 times at San Antonio and 20 times in the Dallas district.

Attempts were made to ascertain what effect temperatures alternating between −29° to −40° and +20° C. had on the viability of urediospores under dry conditions. Sets of spore-dusted slides were prepared, sufficient in number to permit germination tests at the end of 1, 2, 3, 6, 9, 12, and 15 days, respectively. The slides were kept alternately at the subzero temperature −29° to −40° for 24 hours and at 20° for 24 hours, and a control set was kept at −29° to −40°.

For the first 3 days of the experiment there was no significant difference in the survival of urediospores of *Puccinia graminis tritici* form 15 and *P. triticina* when exposed alternately to subzero and above-zero temperatures or to constant low temperatures (table 3). However, after 6 days' exposure to temperatures alternating between −29° to −40° and 20° C., spores of *P. triticina* were more severely

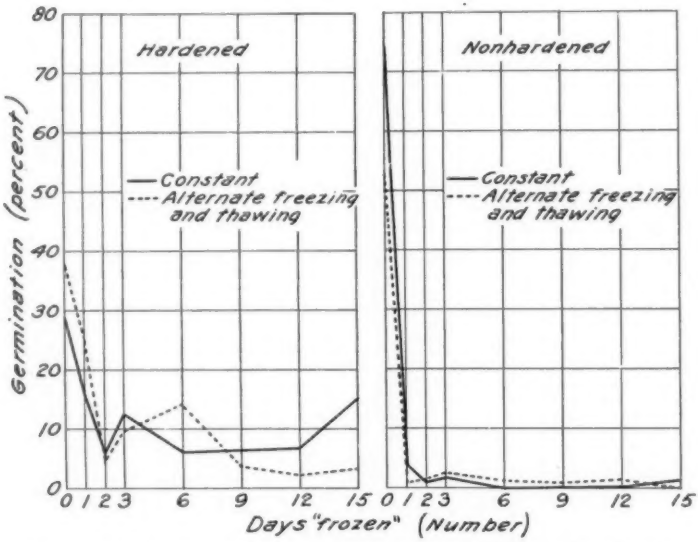

FIGURE 2.—Effect of alternating subzero and above-zero centigrade temperatures on the viability of hardened and nonhardened urediospores of *Puccinia graminis tritici* form 15.

injured than at a constant temperature of −29° to −40°. On the other hand, there appeared to be no consistent differential effect of constant and of alternating temperatures on urediospores of *P. graminis tritici*, except in the case of hardened spores after 9 days (fig. 2), when the spores withstood constant low temperature better than alternating temperatures. In all cases hardened urediospores withstood the low temperature better than nonhardened spores (fig. 2).

Of the spores of *Puccinia graminis tritici* exposed to alternating temperatures, more nonhardened than hardened spores were killed by the first exposure to a subzero temperature. After the marked diminution in percentage of germination during the first 2 days of exposure, there was no perceptible further diminution in hardened

spores until they had been exposed alternately to subzero and above-zero temperatures during a period of 9 days (fig. 2).

TABLE 3.—*Effect of alternate subzero and above-zero centigrade temperatures on viability of hardened and nonhardened urediospores of Puccinia graminis tritici and P. triticina*

Days at −29° to −40° C. alternating with 20° (number)	Percentage of germination [1]				Days at −29° to −40° C. (number)	Percentage of germination [1]			
	P. graminis tritici form 15		*P. triticina*			*P. graminis tritici* form 15		*P. triticina*	
	Hardened	Nonhardened	Hardened	Nonhardened		Hardened	Nonhardened	Hardened	Nonhardened
0	37.6	52.9	55.8	23.9	0	28.9	74.1	56.3	47.7
1	24.9	.7	42.6	3.6	1	16.0	3.8	22.4	10.9
2	4.4	1.3	9.4	2.6	2	5.9	.9	30.8	6.4
3	9.8	2.3	29.9	3.0	3	12.5	1.8	17.6	3.8
6	14.1	1.2	6.5	.4	6	6.1	.0	18.8	2.5
9	3.6	.8	7.8	.0	9	6.5	.3	32.9	11.0
12	2.1	1.3	9.1	.3	12	6.7	.3	27.7	6.6
15	3.1	.0	1.4	.1	15	15.2	1.4	22.5	1.8

[1] Average of 2 trials.

Effect of Temperature on Length of Incubation Period

Stakman and Lambert (*21, p. 374*) concluded that "* * * in the past, there has been a tendency for destructive epidemics to develop in warm growing seasons, and for cool seasons to be comparatively free from rust." Temperature undoubtedly influences the length of the incubation period of rust and therefore influences the number of uredial generations and the consequent increase of inoculum with each successive generation. Four generations during a warm growing season may furnish very large amounts of inoculum, but with only two generations during a cool growing season the increase in inoculum would be appreciably less.

Several investigators have reported results of experiments on the effect of temperature on the incubation period. According to Stakman and Levine (*22, p. 69*), uredia of *Puccinia graminis* may appear 6 or 7 days after inoculation at a temperature of 66.5° to 70° F. Their results show that—

> at low temperatures the development of the uredinia was retarded at the rate of 1 day for every 5 degrees of fall in temperature. Infection resulted at as low temperatures as the host could possibly stand. The spores were rather small, but the difference was not as great as in the case of high temperatures, with moderate temperature as a basis for comparison.

Mehta (*16*) found that uredia of *P. graminis* appeared 21 to 22 days after inoculation at a mean temperature of 40° F., in 14 to 15 days at 50.8°., in 11 to 12 days at 54.1°., and in 8 to 9 days at 66.5°. In 1923 Peltier (*17, p. 49*) concluded as follows regarding *P. graminis tritici* form 9:

> However, plants held at 5° for 9 weeks did not develop any rust until they were transferred to a higher temperature. On the other hand, the incubation period of the rust on plants placed in the 10°, 15°, 20°, 25°, and 30° cases was 18, 16, 11, 8, and 8 days respectively. * * * Plants inoculated at 5° C. and maintained at this temperature for 7 weeks developed no rust until they were transferred to a higher temperature.

The writer made experiments to determine the effect of different temperatures on infection and on subsequent development of the rust. The effect of temperature on the entrance and the establishment of the rust in the host was determined first. Three series of plants were inoculated and placed in incubation chambers for 48 hours at 0° to 1°, 10°, and 20° C., respectively. Then, to determine the effect of temperature on the subsequent development, one-third of each set was placed in a room at 20°, another third at 10°, and the remainder at 0° to 1°. All of the plants were kept constantly in a light of about 304 foot-candles.

Puccinia graminis tritici form 35 and *P. graminis avenae* form 2 caused no infection on plants kept in moist chambers for 48 hours at 0° to 1° C. and subsequently kept at the same or at higher temperatures (table 4). On the other hand, rye plants inoculated with *P. graminis secalis* form 7 and incubated similarly at 0° to 1° produced uredia at the end of 17 days at 10° and after 10 days at 20°. It is possible, of course, that the parasite may have gained entrance into the host after the latter had been placed in the warmer temperatures, but this seems unlikely.

TABLE 4.—*Time required for appearance of uredia of 3 varieties of Puccinia graminis at different temperatures under constant light*

Incubation temperature in—		Time required for first appearance of uredia of—[1]		
Moist chamber (48 hours)	Constant light room	*P. graminis tritici* form 35	*P. graminis secalis* form 7	*P. graminis avenae* form 2
° *C.*	° *C.*	*Days*	*Days*	*Days*
20	0–1	70	44	50
	10	14	13	14
	20	8	8	9
10	0–1	----	54	----
	10	16	16	17
	20	9	8	9
0–1	0–1	----	----	----
	10	----	17	----
	20	----	10	----

[1] Each figure is the average of three trials.

At 10° and 20° C. the urediospores of the above-mentioned forms of *Puccinia graminis* germinate and cause infection readily. A temperature of about 20° C. seems to be the optimum for the entrance and establishment of the pathogene.

The three forms of *Puccinia graminis* mentioned above can develop and produce uredia at 0° to 1°, 10°, and 20° C., if given opportunity to gain entrance and to become established in the host at an appropriate temperature (table 4). However, more time was required for urediospores to appear at the lower temperatures. At 0° to 1°, uredia of *P. graminis tritici* form 35 did not appear until 70 days after inoculation. In another experiment, *P. graminis tritici* form 15 produced urediospores normally within 10 or 11 days at 20° and within 18 days at 10°, whereas at 0° to 1° uredia failed to appear within 80 days. This indicates that physiologic forms of *P. graminis tritici* differ in their ability to develop at temperatures just above freezing.

The results suggest that in nature the mycelium may remain dormant in infected plants during the winter if the host tissues remain alive. If this happens, urediospores may be produced as soon as the weather becomes warm; and, if there are susceptible hosts nearby, infection occurs. Several weeks of cool weather then may follow infection and the mycelium may lie dormant until the temperature again rises. Then the uredia may "miraculously" appear, furnishing early inoculum to infect young grain plants and start an early epidemic.

Influence of Temperature on Development of Mycelium within Host Tissue

Some field observations have been made on the survival of stem rust mycelium from one season to another. Lambert (*12*) reports that at Dallas, Tex., the host leaves bearing old pustules were killed by alternate freezing and thawing. Mehta (*16*) states the following conclusions from his observations:

> It is clear that the mycelium of black rust can survive milder weather and but for the absence of the source of infection, it might appear on the cereal crops in the earlier part of spring, if weather conditions are favorable.

Obviously, the host tissue must survive the winter if the rust mycelium is to survive. The question then arises as to whether the mycelium of *Puccinia graminis* can withstand as low temperatures as the host. The following experiments accordingly were made.

Three sets of wheat differentials were inoculated with urediospores of *Puccinia graminis tritici* form 15, incubated in a moist chamber at 20° C. for 48 hours, and then placed in the 0°, 10°, and 20° chambers, respectively. This experiment was repeated three times. Uredia developed on plants placed at 10° and 20°, but at the end of 60 days none had developed at 0°. Plants in the 0° room were removed to the greenhouse at the end of 40, 50, 60, 70, 80, and 90 days, respectively. On plants kept at 0° to 1° for 80 days and then transferred to 20°, uredia appeared within 8 days after the transfer, but none appeared on plants kept at 0° to 1° for 90 days.

The mycelium of *Puccinia graminis avenae* form 2 lived for 80 days at 0° to 1° C. without producing uredia, but they appeared 4 days after the infected plants were removed to the greenhouse.

In another experiment, seedling wheat plants, inoculated with *Puccinia graminis tritici* form 15 and kept in moist chambers at 20° C. for 48 hours, were placed in the 10° room under constant light and allowed to remain there until flecks developed. Then they were removed to the 0° to 1° room, and 32 days later uredia appeared.

To determine whether the mycelium of *Puccinia graminis* can withstand as low temperatures as the host plant, seedling plants of hardy winter varieties were inoculated, kept in moist chambers for 48 hours, and then placed in the greenhouse. When flecks appeared, the plants were placed in the 0° C. chamber for at least 10 days to allow them to harden, after which different sets were kept at −12°, −10°, −7°, and −5°, respectively, for 24 hours. The same procedure was followed with plants in tillering and boot stages. After freezing, the plants again were placed at 0° for 24 hours to allow them to "thaw out" gradually. They then were removed to the greenhouse and were watched for the appearance of stem rust. Each of the experiments was repeated several times.

Uredia of *Puccinia graminis tritici* form 35 appeared on Minhardi wheat in the greenhouse about 3 days after having been subjected to −5° C. for 24 hours. In a later experiment, uredia of *Puccinia graminis tritici* form 15 appeared on Kanred and Minturki wheats in 3 and 6 days, respectively, after having been subjected to −10° for 24 hours. In this experiment low temperature seemed to affect the organism more in plants of Minturki wheat than in those of Kanred wheat, as it took the uredia 3 days longer to appear. In subsequent tests at −13° the infected leaves were killed. Uredia of *Puccinia graminis secalis* form 7 appeared on plants of Petkus rye that had been exposed to −7° for 24 hours.

It is clear that at low temperatures, the rust mycelium can remain dormant for long periods in the host, and it apparently can withstand as low a temperature (−10° C.) as the host plants. However, lower temperatures must be tried to demonstrate the latter point conclusively.

Effect of Temperature on Infection Type

Stakman and Levine (*22*, *23*) and Levine (*13*, *14*) have shown that environmental conditions unfavorable for the host are unfavorable also for the parasite, affecting the virulence and spore size of the latter. Waterhouse (*25*, *p. 676*) recently reached the following conclusion from his experiments:

> In the case of *Puccinia graminis tritici* there was change from complete susceptibility in the hotter months to complete resistance in the cooler part of the year. The reactions on several of the differential hosts showed this change. Some physiologic forms show much more marked alterations of this nature than do others. Changes from summer to winter conditions alter the reactions shown by *Puccinia graminis avenae* on "Joanette" from complete susceptibility to complete resistance.

The writer made a study of the effect of temperature on the infection type. Even at 0° C. some rust appeared on plants that had previously been inoculated and kept for 48 hours in a moist chamber at 20°, but the uredia were very small. There was no significant difference in infection types at 10° and 20°, the only noticeable difference being a higher proportion of type 3 to type 4 at 10 °. However, not only were much smaller pustules produced by several physiologic forms at 0° to 1° than at 20°, but the areas surrounding the pustules were strongly etiolated. The uredia thus developed resembled type 1 as long as the plants remained at 0° to 1°, but they changed to type 3 or type 4 when the plants were placed at 20°. For example, Little Club wheat at 20° is moderately to completely susceptible to *Puccinia graminis tritici* form 35, infection types 3 and 4 ordinarily being produced; but at 0° to 1° pustules approaching type 1 were produced 70 days after inoculation in 2 out of 3 trials (fig. 3). In a later experiment, seedlings of Little Club, inoculated on February 27 with spores of *Puccinia graminis tritici* form 35, were placed at 0° to 1° on March 6. Thirty days later uredia similar to type 1 appeared.

A set of differential varieties of wheat, inoculated with *Puccinia graminis tritici* form 15 and kept at 10° C. until flecks appeared, was placed in the 0° to 1° chamber. After 32 days, pustules of "type 1",[5] instead of the characteristic type 3 or 4, appeared on 11 of the differential hosts (fig. 4). The first leaves of plants on which the apparent

[5] Small pustules approaching type 1 are designated "type 1."

type 1 pustules were produced at 0° to 1° were cut off, and new leaves were allowed to develop at 20°. They then were inoculated with spores of form 15, and this time the plants were allowed to remain at 20°. Normal vigorous pustules of types 3 and 4 resulted (fig. 4).

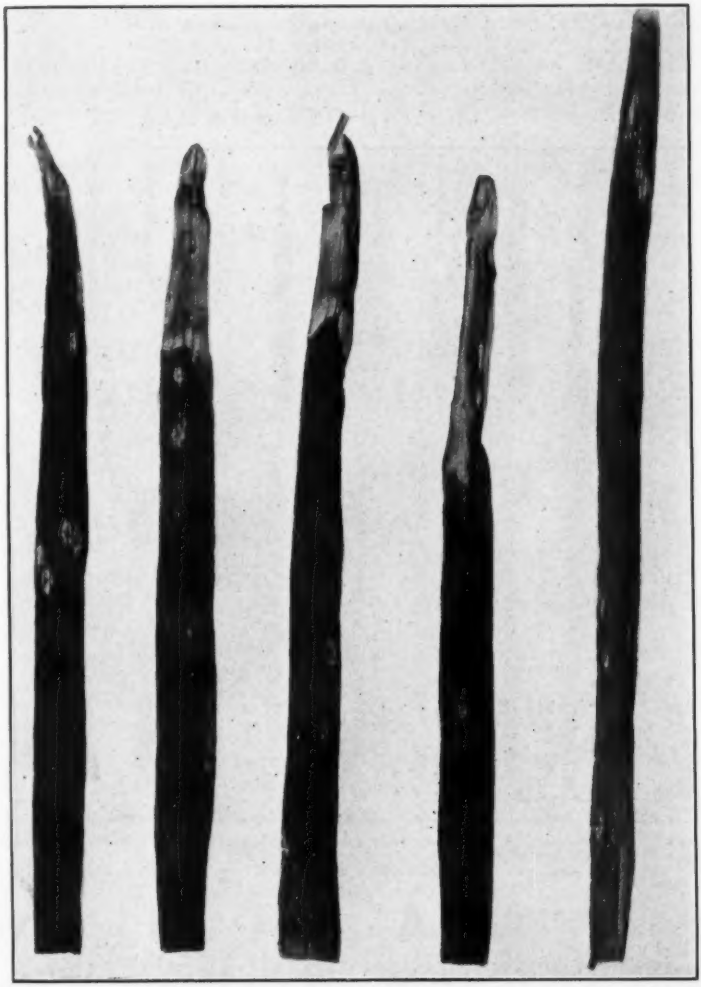

FIGURE 3.—"Type 1" uredia produced on Little Club wheat by *Puccinia graminis tritici* form 35 at 0° to 1° C. This form normally produces uredia of types 3 and 4 on Little Club wheat at 20°.

Inoculation with spores from the type 1 pustules, produced at 0° to 1° on Vernal emmer, resulted in the development of type 4 uredia when cultured at 20°. Type 1 pustules of form 15, previously formed at 0° to 1°, developed into type 3 after the plants were removed to

20°. *Puccinia graminis secalis* form 7 and *Puccinia graminis avenae* form 5 produced normal uredia even at 0° to 1°. The type 1 uredia, therefore, represented merely the lower limit of development of rust under abnormal conditions.

Difference in Ability of Different Physiologic Forms to Develop at 0° to 1° C.

Physiologic forms of *Puccinia graminis* seem to vary in their ability to develop at low temperatures. This is shown by the following experiment. On February 3, 1930, 10 pots of Little Club wheat were

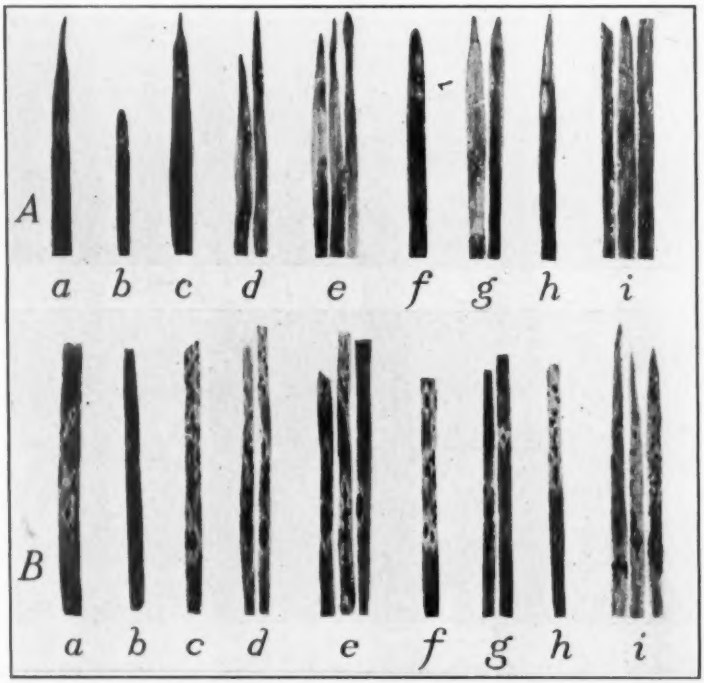

FIGURE 4.—*A*, Minute uredia (type 1) produced by *Puccinia graminis tritici* form 15 on varieties of *Triticum* spp. at 0° to 1° C.; *B*, normal development of rust at about 20° on secondary leaves of plants shown in *A*. *a*, Marquis; *b*, Kanred; *c*, Arnautka; *d*, Mindum; *e*, Spelmar; *f*, Kubanka; *g*, Acme; *h*, einkorn; *i*, Vernal emmer.

inoculated with *P. graminis tritici* form 15 and 10 with form 36. The plants were kept in moist chambers under the same conditions for 48 hours and then placed in the same greenhouse for 4 days. At the end of this period (Feb. 9) they were placed at 0° to 1° C. under constant light. Uredia of form 36 appeared on February 21, and only one uredium of form 15 had appeared by March 18, while form 36 had sporulated and produced numerous normal uredia (fig. 5). Pustules of *P. graminis tritici* form 15, however, appeared shortly after the plants again were placed in the greenhouse, showing that the mycelium

had remained alive at 0° to 1°, although there had been no appreciable development.

Two forms of *Puccinia graminis avenae* (forms 2 and 5) seemed also to develop at different rates at 0° to 1° C. For example, on February 3, 1930, several pots each of Victory oats were inoculated with these forms and kept in moist chambers under the same conditions. After 48 hours they were removed to the same greenhouse and kept 4 days. On February 9 they were placed at 0° to 1° under constant light. Uredia of form 5 appeared on February 22, while uredia of form 2 did not appear until March 3.

Evidently there are differences in the ability of physiologic forms of *Puccinia graminis tritici* and *P. graminis avenae* to develop at 0° to 1° C., whereas at 20° there are no apparent differences.

Form 7 of *Puccinia graminis secalis* developed well at 0° to 1° C., almost as well as at 10° and 20°, perhaps accounting for the prevalence of rye rust in the later fall in northern regions.

Low temperatures seem to stimulate the development of telia, because this stage of *Puccinia graminis secalis* form 7 formed abundantly

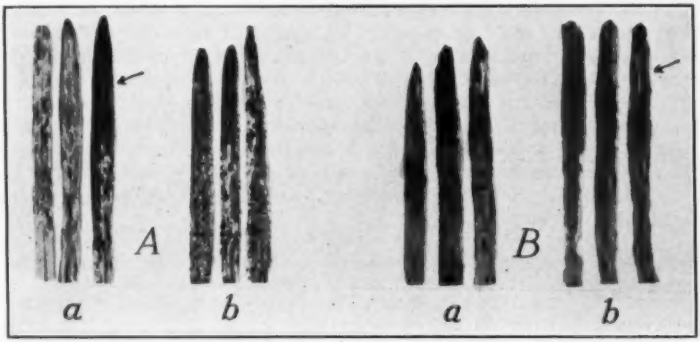

FIGURE 5.—Variation in ability of physiologic forms of *Puccinia graminis* to produce uredia at 0° to 1° C. *A*, Leaves of Little Club wheat seedlings infected with *P. graminis tritici: a*, Form 15; *b*, form 36. *B*, Leaves of Victory oat seedlings infected with *P. graminis avenae: a*, Form 5; *b*, form 2.

at 0° to 1° C. *P. graminis tritici* form 15 and form 36 produced abundant telia after the infected plants had been transferred from 0° to 1° to a warm greenhouse, where telia do not ordinarily form in abundance.

EFFECT OF LIGHT INTENSITY

The effect of light on certain rusts has been studied by several investigators. Fromme (*7, p. 519*) found that "Total light exclusion either early or late in the incubation period checks the development of *P. coronifera* and results in an almost complete cessation of growth." Mains (*15*) studied the nutritional phase and arrived at the following conclusions: "When the host is deprived of carbohydrate, *P. coronata* and *P. sorghi* require light in order to develop. However, starvation will not bring about immunity." Stakman and Piemeisel (*24*) pointed out that a good deal of sunlight is necessary for the best development of stem rust. They state (*24, p. 487*):

> During periods of cloudy weather, however, the incubation period may be lengthened a week or more, and the rust does not develop so abundantly as

during bright weather. * * * The shaded plants were more weakly infected than the others. Partially etiolated plants were infected with difficulty and the rust developed very weakly on them.

Peltier (*18*) concluded that light is not a factor in the initial infection of wheat by stem rust, but it is essential for subsequent development. Gassner (*8*) also pointed out that light governs rust development in proportion to the effect it has on the metabolism of the host plant and that high light intensities tend to shorten the incubation period.

The writer made experiments with *Puccinia graminis* to determine the effect of different light intensities, at uniform temperature and humidity, on length of incubation period, type of infection, and morphology of urediospores.

Three sets of differential varieties of wheat and oats, after being inoculated and kept in moist chambers for 48 hours under uniform conditions, were placed in the 20° C. chamber under light intensities of 394, 301, and 162 foot-candles, respectively.

Under all degrees of light used the plants were much lighter in color than those grown under natural light in the greenhouse. There was slight etiolation under 394 and 301 foot-candles and considerable etiolation under 162 foot-candles. In general, oats did not thrive so well as wheat in artificial light, and plants of both cereals became very weak and etiolated after about 3 weeks. Moreover, root rot seriously hindered the growth of the plants, greater damage resulting under the light intensity of 162 foot-candles than under the higher intensities. Marquis wheat was least affected, while the durum varieties Arnautka and Spelmar were heavily infected at low light intensities. Rye thrived under artificial light for a period of about 3 weeks, after which the plants died rapidly.

Effect of Different Light Intensities on Length of Incubation Period

High light intensities shortened the incubation period appreciably. Under an intensity of 394 foot-candles, uredia of *Puccinia graminis tritici* form 15 appeared in approximately 9 days, while under the lower intensities of 301 and 162 foot-candles 10 to 11 days were required. There were no consistent differences in incubation periods in different varieties of *Triticum* spp. Uredia of *P. graminis avenae* form 2 appeared in 8 to 9 days under 394 foot-candles, while 9 to 12 days were required for their appearance under the lower light intensities.

Effect of Different Light Intensities on Type of Uredia Produced

A study was made of the effect of different light intensities on the infection types produced by *Puccinia graminis avenae* form 2 on 4 differential oat varieties and by *P. graminis tritici* form 15 on 11 differential wheat varieties. In addition, a comparative study was made of the effect of the same light intensities on the infection types produced by three varieties of *P. graminis*, namely, *P. graminis tritici* form 35 on Little Club wheat, *P. graminis secalis* form 7 on Petkus rye, and *P. graminis avenae* form 2 on Victory oats.

The results indicate that the different degrees of light used caused no significant or permanent changes in type of infection, although deficient light causes pustules to be considerably smaller.

From these experiments it may be concluded that under low light intensities the normal development of stem rust pustules is inhibited, resulting in the production of smaller pustules, but there is no indication of a significant or permanent change in type of infection; there is no indication that certain hosts that are susceptible under intense light are rendered resistant under low light.

Effect of Different Light Intensities on Size and Shape of Urediospores

Urediospores produced under the various light intensities were measured and compared with those produced under sunlight at 20° C. Some similar studies had been made by Levine (*13*), who found that urediospores produced in deficient light were shorter than normal.

Table 5.—*Variants and constants for dimensions of urediospores of Puccinia graminis tritici form 15 grown under different light and temperature conditions in the greenhouse*

LENGTH

Cultural conditions		Range of variability	Constants		
Temperature (°C.)	Light		Mean	Standard deviation	Coefficient of variability
	Foot-candles	μ	μ		
10	334	27.83–46.46	35.50±0.19	4.01±0.14	11.29±0.39
20	394	26.91–43.47	35.19± .16	3.39± .11	9.63± .32
	301	25.53–45.08	36.13± .17	3.54± .12	9.80± .33
	162	27.60–42.78	34.41± .16	3.42± .12	9.94± .34
	(1)	25.76–44.85	34.54± .17	3.54± .12	10.24± .35

WIDTH

10	334	17.94–27.14	22.03±0.07	1.56±0.05	7.09±0.24
20	394	18.86–27.14	22.11± .07	1.39± .05	6.27± .21
	301	19.55–27.60	21.90± .07	1.55± .11	7.07± .24
	162	17.25–25.76	21.77± .08	1.70± .06	7.80± .26
	(1)	19.32–27.60	23.03± .07	1.52± .05	6.59± .22

RATIO OF LENGTH TO WIDTH

		Correlation coefficient			
10	334	−0.107±0.047	1.637±0.011	0.23±0.01	14.29±0.48
20	394	− .239± .045	1.621± .010	.21± .01	12.79± .43
	301	− .255± .045	1.678± .011	.22± .01	13.26± .45
	162	− .197± .046	1.621± .011	.22± .01	13.80± .47
	(1)	− .242± .045	1.427± .010	.20± .01	14.20± .48

[1] Sunlight.

In this study the length and width of 200 spores from each of the different cultural conditions were measured at random on a slide. Because the differences were not great, it was necessary to employ statistical methods to make comparisons (tables 5 and 6). There was negative correlation between length and width of spores in all lots measured. Spores produced under 301 foot-candles were significantly longer but not significantly wider than those under 162 foot-candles, and they were significantly longer but narrower than those produced in sunlight at 20° C., and as a result of this differed

from the latter also in shape, i. e., in ratio of length to width. Spores produced under 394 foot-candles were narrower, though not significantly longer, and quite different in shape from those produced in sunlight at 20°. Those produced under 162 foot-candles were narrower than spores produced in sunlight at 20° and differed from them in shape. Spores produced under 334 foot-candles at 10° did not differ significantly from those produced under 394 foot-candles at 20°.

TABLE 6.—*Differences between means of dimensions of urediospores of Puccinia graminis tritici form 15 cultured under different light and temperature conditions*

Cultural conditions compared	Length		Width		Ratio of length to width	
	Difference in means	Difference divided by P. E.	Difference in means	Difference divided by P. E.	Difference in means	Difference divided by P. E.
	μ		μ			
334 foot-candles at 10° with 301 foot-candles at 20°	0.63	2.46	0.13	1.31	0.041	2.66
394 foot-candles at 20° with 301 foot-candles at 20°	.94	4.01	.21	2.12	.057	3.93
394 foot-candles at 20° with 162 foot-candles at 20°	.78	3.40	.34	4.23	.000	.00
301 foot-candles at 20° with 162 foot-candles at 20°	1.72	7.35	.13	3.20	.057	3.73
394 foot-candles at 20° with sunlight at approximately 20°	.65	2.78	.92	9.29	.194	13.96
301 foot-candles at 20° with sunlight at approximately 20°	1.59	6.66	1.13	11.41	.251	17.43
162 foot-candles at 20° with sunlight at approximately 20°	.13	.56	1.26	11.85	.194	13.20

DISCUSSION AND CONCLUSIONS

The results obtained show that the death of urediospores in the winter probably is not always due to low temperatures as such. This is shown by the fact that some urediospores of the *tritici* and *phlei-pratensis* varieties of *Puccinia graminis* remained viable after having been exposed for 45 days to temperatures ranging from −29° to −40° C. It is true that a large percentage of urediospores usually are killed within the first 24 hours after being exposed to the low temperature, but most of those that survive retain their viability for a long time.

Hardened urediospores are more likely to survive than those not hardened. Spores produced under normal conditions were hardened at 0° C. for 10 days or longer, and their viability was compared with that of similarly produced, nonhardened spores. The hardened spores were more resistant to cold than the nonhardened. It seems probable, therefore, that spores formed at low temperatures in the fall may be more cold-resistant than those formed at higher temperatures.

Not only are some urediospores able to withstand continuous low temperatures, but a few may withstand fluctuating temperature in a surprising manner, especially when dry. It seems quite likely, therefore, that the death of urediospores in nature during the northern winters is due neither to low temperature nor to alternating low and moderate temperatures when the spores remain relatively dry, but rather to alternate freezing and thawing during wet weather.

Uredial mycelium apparently can withstand temperatures as low as those endured by the host plants. For example, mycelium remained viable within infected wheat plants kept for 24 hours at −10° C. When the plants were again placed at a temperature of approximately 20°, rust pustules developed within a few days. The mycelium within infected rye plants withstood a temperature of −7° for 24 hours and subsequently produced pustules. In nature, therefore, the mycelium probably can survive in any plant tissues that remain alive during periods of cold weather. The experiments indicate also that this mycelium can subsequently produce urediospores with the advent of favorable temperatures. This is shown by the fact that both wheat and oats infected with rust were kept for 80 days at a temperature of 0°, during which time no uredia appeared; when the plants were transferred to the greenhouse, however, uredia appeared in 8 days.

Temperature also has a profound effect on the development of rust in normal plants after infection once has taken place. For example, it required approximately a week longer for uredia to appear at 10° C. than at 20°. According to this, for every drop of 1° in temperature the incubation period would be lengthened about two-thirds of a day. This probably is not an absolute rule, as there is considerable variation in the effect of temperature on the incubation period. The generalization would be approximately true only for a range of temperatures in which the rust could develop fairly well. The fact, however, is very significant, because, in the spring wheat area of the United States, there is a relatively short time in which rust develops. Usually it is only about 45 days. At high temperatures, therefore, about 6 crops of urediospores would be produced during the season, while at low temperatures only 2 or at most 3 successive crops would be produced, and the amount of inoculum and subsequent development of rust would be greatly reduced. Field observations indicate that relatively slight differences in temperature during the critical period for the development of rust, other things being equal, determine the severity of a rust epidemic. In many years, there is a very close race between grain crops and rust. The greatest damage usually occurs just before harvest; and in many seasons the crops escape severe damage if the rust parasite is unable to produce a large amount of inoculum at that time.

Excessively low temperatures may greatly lengthen the incubation period. For example, in some experiments it required 70 days for form 35 of *Puccinia graminis tritici* to produce uredia at 0° to 1° C., and some physiologic forms seem to be entirely unable to produce uredia at so low a temperature. It is likely that the effect of temperature may vary with the particular physiologic forms concerned in the development of rust in a given locality or in a given season.

Temperature is important not only in governing the length of the incubation period and the consequent amount of inoculum produced in a given season but also in determining the size of uredia and consequently the number of spores on each infected plant. This is clearly shown by the fact that at 0° to 1° C. *Puccinia graminis tritici* forms 15 and 35 produced pustules resembling type 1 pustules, which are extremely small, whereas these same forms produced type 4 pustules on the same variety at higher temperatures. The small pustules, however, may enlarge if the temperature rises. Tempera-

ture therefore determines the amount of inoculum in two ways: (1) by determining the number of urediospore generations in a given season and (2) by determining the number of spores per pustule.

The ability of certain physiologic forms of *Puccinia graminis* to develop at low temperatures may be important from the standpoint of regional epidemics of stem rust in the spring wheat area. If inoculum of these forms is present in this area early in the grain-growing season, stem rust can increase regardless of cool weather. This complicates the stem rust problem further, because low temperature retards the development of many forms of *Puccinia graminis tritici* sufficiently to inhibit severe infection.

As temperature extremes seem to induce early teliospore formation, teliospores are likely to be produced in nature when it is either abnormally hot or abnormally cool and when the potential danger of rust is consequently reduced. However, the effect of moisture must not be overlooked in this connection.

Light, of course, affects rust development. However, in the experiments made by the writer, the differences in light intensity were not sufficiently great to change the host reaction from susceptible to resistant or vice versa. There were fluctuations, but a type 4 infection was never reduced to a type 1 or a type 2 by withholding light. It seems unlikely that in nature there would be sufficient variation in total light during any given season to affect appreciably the development of rust.

The experiments indicate that temperature is the most important factor governing the development of rust in a susceptible host, assuming, of course, that there is sufficient moisture for infection and enough light for normal growth of plants. The stem rust pathogene seems to be well equipped to withstand vicissitudes, for it is able to persist at a very low temperature. However, its development to epidemic proportions is conditioned by temperature, as pointed out by Stakman and Lambert (*21*).

SUMMARY

Hardened urediospores of *Puccinia graminis* withstood subzero centigrade temperatures better than nonhardened urediospores. Hardened urediospores of wheat stem rust (*Puccinia graminis tritici*) and of timothy stem rust (*Puccinia graminis phlei-pratensis*) were able to withstand subzero temperatures for at least 45 days and hardened urediospores of oat stem rust (*Puccinia graminis avenae*) for 40 days. A few nonhardened urediospores of wheat stem rust withstood 45 days of subzero temperature; timothy stem rust, 35 days; and oat stem rust, 15 days.

Temperatures alternating above and below 0° C. had no more killing effect on nonhardened urediospores of *Puccinia graminis tritici* form 15 than did constant subzero temperatures. On the other hand, alternating temperatures killed more hardened spores after 9 days than did constant subzero temperatures.

The incubation period of stem rust is lengthened by low temperature. It took approximately a week longer for uredia to appear on plants kept at 10° C. than at 20°. Uredia developed at 0° to 1°, but much more time was required. *Puccinia graminis tritici* form 35 produced uredia in about 70 days at 0° to 1°. When the infected

plants were placed at 0° to 1° 48 hours after inoculation, form 15 produced no uredia during 80 days' exposure at this low temperature.

The mycelium of at least some physiologic forms of stem rust can withstand as low temperatures as the host. This was true of *Puccinia graminis tritici* form 35 and *Puccinia graminis secalis* form 7.

Except under extreme conditions, temperature has little effect on the type of uredia produced. At 10° C. smaller pustules were produced than at 20°. A temperature of 0° to 1° effected an apparent change of infection type of *Puccinia graminis tritici* forms 15 and 35. An infection type similar to type 1 was produced at 0° to 1° in place of type 3 or 4, normally produced at 20°. However, after the infected grain plants were taken from 0° to 1° and placed in the greenhouse at 20°, the type 1 infection developed into type 3. Therefore this temporary change at 0° to 1° was due to low temperature.

Physiologic forms of *Puccinia graminis tritici* and *Puccinia graminis avenae* differed in ability to produce uredia at low temperatures. At 0° to 1° C., *Puccinia graminis tritici* form 36 produced normal uredia readily; forms 15 and 35 produced only a few minute pustules similar to a type 1 infection; *Puccinia graminis avenae* form 2 did not produce uredia as readily as form 5. *Puccinia graminis secalis* form 7 also produced normal uredia at 0° to 1°.

A temperature of 0° to 1° C. stimulated the production of telia.

Different light intensities had no significant effect on the infection type produced, but low light intensities delayed the appearance of uredia. It took 1 to 2 days longer for uredia of *Puccinia graminis tritici* form 15 and *Puccinia graminis avenae* form 2 to appear under light intensities of 301 or 162 foot-candles than under a light intensity of 394 foot-candles.

Light intensities and quality of light seemed to affect the size and shape of urediospores. Urediospores of *Puccinia graminis tritici* form 15, formed under a light intensity of 301 foot-candles at 20° C., were significantly longer than those produced under other light conditions. Urediospores produced under artificial light were significantly longer and narrower than those produced in the greenhouse.

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A NEW CRITERION FOR THE COMPARISON OF TOXICITY WITH RESPECT TO CONCENTRATION AND TIME[1]

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INTRODUCTION

In toxicological studies of rotenone and related compounds (*18*, *19*, *20*) [2] the method (*17*) used by the writer required the determination of the survival times of the test animal, the goldfish, in a number of concentrations of the toxic substance. Variables other than survival time and concentration, such as the physiological phenomenon to be used as the end point and the method of measuring it, size of the test organism, and temperature, were eliminated from consideration by restricting them to constant values in all the tests. These values were determined by practical conditions; thus, the criteria were the death point, the arithmetic average of at least 10 individuals weighing from 2.0 to 2.5 g, and a temperature of 27° C. This restriction to two variables permitted the course of toxic action under these conditions to be plotted graphically as a curve in two dimensions, and the range of concentrations used was such that the resultant curve became parallel, or nearly parallel, to both axes. Such a curve or its companion, the concentration versus reciprocal-of-survival-time curve, may serve for the derivation of a value that will express the relative toxicity of the substance, this value being obtained either from the entire curve or from certain portions of it.

CRITERIA OF OTHER WORKERS

In studying such curves a number of investigators have attempted to formulate an equation that would express the relationship between concentration and time (*1* to *4*, *6* to *16*, *22* to *57*). An equation formulated by Glaser (*22*) fits the data obtained by the writer better than any other. In a study of the relationship between concentration and time of action in which tadpoles were paralyzed with hydrocyanic acid, Glaser obtained a curve resembling an equilateral hyperbola. To express this relationship accurately he developed the following equation: $(C-b)\ (1-e^{-a(t-g)})=k$, in which C is the concentration, b is a concentration-tolerance factor (a constant), g is a time-tolerance factor (a constant), e is the base of natural logarithms, a is a proportionality factor (velocity of diffusion), t is the time, and k is a constant. The use of this equation, however, entails much experimental work as well as mathematical computation, as it requires the determination of 4 constants, 2 of which are the asymptotes of the curve.

There is still another objection to the use of Glaser's formula as a means of measuring relative toxicity. Although a value might be obtained representing the complete action of the toxic substance (as

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[2] Reference is made by number (italic) to Literature Cited, p. 888.

the k of an equation expressing this action), it would be difficult to interpret its significance when used as a measure of relative toxicity. It might be comprehensible if a change in toxicity were accompanied by a shift in the two tolerance factors (that is, the two asymptotes) of the same order of magnitude and direction, but this is not necessarily so. In the studies made by the writer the greatest disproportion appears in the comparison of the toxicity of nicotine (*21*) with that of rotenone. Compared according to the magnitude of the thresholds (the concentration-tolerance), rotenone is 800 times as toxic as nicotine, but compared according to the minimum time in which goldfish may be killed in solutions of these substances (the time tolerance) nicotine is more than 6 times as toxic as rotenone. The inadequacy of expressing relative toxicity by a single magnitude which will include the relationship of these parameters is therefore apparent.

Because the use of the entire curve involves such difficulties, for practical purposes it is desirable to find some portion of it that will suffice for a simpler comparison. In previous papers (*18, 19, 20, 21*) it was emphasized that there are serious objections to the comparison of the survival times at a given concentration, to the comparison of the concentrations necessary to produce death (or any other physiological phenomenon) in an arbitrary time, or to the comparison of the concentrations necessary to just cause death (threshold of toxicity concentration). If comparisons are based on the location of a point on the curve, this point should always fall on the arch of the curve, the region in which the proportional changes in the two variables, concentration and time, do not differ greatly. On either side of this region the curve flattens out so quickly that a toxic value for a point falling on either limb emphasizes one variable to the exclusion of the other; that is, on the one hand a point rapidly nears the threshold of toxicity, the lowest concentration at which the substance will have any toxic (lethal) effect, regardless of the time necessary to kill, and on the other hand a point rapidly nears the minimum time required to kill, regardless of the concentration. For these reasons the writer has used the Powers formula for the expression of relative toxicity. Powers (*41*) disregarded the two extremes of the curve and expressed relative toxicity by a formula dependent only on data falling within the portion of the curve approximating an equilateral hyperbola. In the concentration versus reciprocal survival-time curve (the velocity-of-fatality curve) this portion approximates a straight line (the theoretical velocity-of-fatality curve) which cuts the x axis at a point a, designated the theoretical threshold of toxicity concentration, and makes an angle, designated θ, with this axis. Thus two values are obtained, the threshold value and the slope of the theoretical velocity of fatality. Powers expressed the reciprocal relation of these factors mathematically by the equation, toxicity $=\sqrt{\frac{\tan \Theta}{a}}$

In a later publication (*42, p. 98*) Powers states:

> Perhaps a better and more simple expression for the relative toxicity is the equation of the theoretical velocity of fatality curve itself, which is the equation of a straight line. That is, $y/(x-a)=k$, where y=the velocity of fatality [the reciprocal of the survival time], x=the concentration of the solution tested, and a=the theoretical threshold of lethality concentration.

k is a constant representing the relative toxicity of the substance. The value of k is, of course, the slope of the straight line representing the theoretical velocity of fatality.

The curves obtained by the writer differed from those obtained by Powers in two respects, and therefore the use of the formula was discontinued. In the first place, Powers, using substances of low toxicity, salts of the alkali and alkaline earth metals, obtained survival-time curves that were not far removed from the x axis (the concentration axis) as compared to the y axis, whereas the writer, using highly toxic substances, obtained curves with larger and significantly different minimum times of kill. Thus, for the rotenone derivatives the Powers formula, which emphasizes two parameters, the threshold of toxicity and the maximum rate of increase of the velocity of fatality, without considering this third significant parameter, seems to be neither sufficiently inclusive nor impartially exclusive.

If this third parameter, the toxicity at high concentration (the minimum survival time or the value of the horizontal asymptote) is introduced into the formula $\sqrt{\frac{\tan \Theta}{a}}$, as t_0, it would logically appear in the denominator—that is, the formula would read $\sqrt{\frac{\tan \Theta}{at_0}}$,—and the value obtained would vary directly with the slope of the theoretical velocity-of-fatality curve and inversely with the threshold concentration and with the minimum survival time, as it should. Also, the value would now be really the square root of a square and expressible in rational units. The use of this formula for the comparison of toxicity, however, entails the determination of the asymptotes, which can be made accurately only with great difficulty.

In the second place, Powers obtained velocity curves that were sigmoid; therefore, the theoretical velocity-of-fatality curve, because it was a straight line passing through the point of inflection, was drawn with no great difficulty. However, the initial curvature, concave upwards, was not indicated in the tests with the rotenone derivatives, this possibly being due to the great toxicity of these compounds. Therefore, a straight line approximating this portion of the curve was much more difficult to locate.

The latter difficulty still obtains in the use of the slope of the theoretical velocity-of-fatality curve, that is, the equation of the straight line as suggested later by Powers (*42*). However, the slope would give relative toxic values based on the second parameter only, which is desirable for practical purposes. The writer is concerned here with the problem of finding the comparative toxicities of the substances at their greatest efficiencies with respect to concentration and time, that is, the range in which a quick action is commensurate with a low concentration, and not with the question of the minimum concentrations at which they just become toxic in any time no matter how great, or the minimum time it takes to kill in any concentration no matter how great. Although Powers' theoretical velocity of fatality corresponds to the portion of the survival-time curve showing this greater efficiency (the arch of the curve), in the case of rotenone and its derivatives the straight line corresponds rather to the entire

left portion of the curve to about the middle of the bend. Thus, the slope of the straight line as a criterion for the expression of relative toxicity, although nearer what is desired, is still not entirely satisfactory.

Campbell (*5*) suggests that the relative toxicity may be expressed numerically as an "effect comparison" by the ratio of the areas under the velocity-of-fatality curves and as a "dosage comparison" by the ratio of the areas between the curves and the y axis. Such comparisons, however, involve the use of arbitrary upper limits on the axes.

MINIMUM *ct* PRODUCT AS A CRITERION

Examination of the survival-time curves obtained with the rotenone derivatives with regard to the possible approximation of their equations with the asymptotic equation of the hyperbola shows two important differences. They are not asymptotic to the coordinate axes, and the *ct* product (the product of concentration and survival time), instead of being constant, decreases as a point moves from either extreme of a curve toward the arch and reaches a minimum value somewhere on this arch, as shown in table 1. When the curves are referred to their asymptotes as axes (that is, to c_0 and t_0, which may be called the concentration tolerance—equivalent to Powers' a—and the time tolerance) they still are not rectangularly hyperbolic, for the product $(c\text{-}c_0)$ $(t\text{-}t_0)$ varies in the same way as does the *ct* product.

TABLE 1.—*Variation of the constants of the asymptotic equations of the hyperbola as shown in tests with rotenone at 27° C.*

[c_0=0.012 mg per liter; t_0=40 minutes]

c	t	ct	$(c\text{-}c_0)$ $(t\text{-}t_0)$	c	t	ct	$(c\text{-}c_0)$ $(t\text{-}t_0)$
Milligram per liter	*Minutes*	*Milligram minutes per liter*	*Milligram minutes per liter*	*Milligram per liter*	*Minutes*	*Milligram minutes per liter*	*Milligram minutes per liter*
4.0	46	184	23.9	0.075	115	8.63	4.73
3.0	46	138	17.9	.055	145	7.97	4.52
2.0	48	96	15.9	.050	155	7.75	4.37
1.0	51	51	10.9	.045	172	7.74	4.36
.50	57	28.5	8.30	.040	194	7.76	4.31
.30	63	18.9	6.62	.035	250	8.75	4.83
.20	70	14.0	5.64	.025	590	14.75	7.15
.10	95	9.50	4.84	.015	2,400	36.0	7.08

It occurred to the writer that for practical purposes one of these minimum products might serve as a criterion for expressing relative toxicity. The equation $(c\text{-}c_0)$ $(t\text{-}t_0)=k$ approximates a somewhat wider range than does the equation $ct=k$, as shown in table 1, and so might be more acceptable. To use the minimum product of $(c\text{-}c_0)$ $(t\text{-}t_0)$, however, would entail the difficulty of determining the asymptotes. It is conceivable that more than one compound might have the same minimum *ct* product and yet be different in toxicity, as shown by the different positions of the arches of their survival time curves, but insofar as is indicated by the compounds studied, this difference is not likely to be great. This problem is also encountered in other arithmetic comparisons of toxicity.

The meaning of a toxic value based on the minimum *ct* product is disclosed by a study of the graphs in figures 1 and 2, in which are

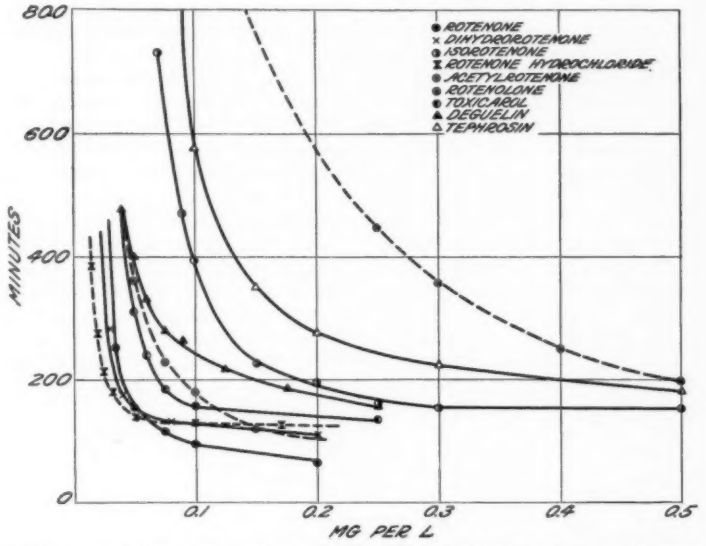

FIGURE 1.—Survival-time curves. Broken curves indicate the use of a lot of fish differing in resistance from the other lots used.

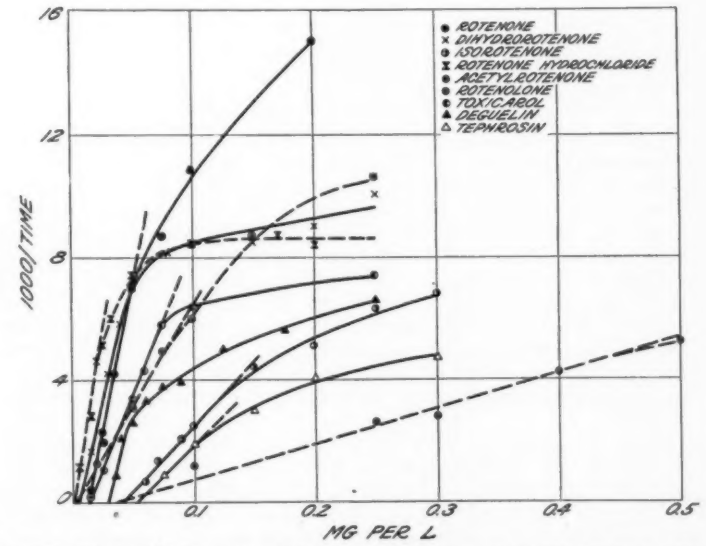

FIGURE 2.—Velocity-of-fatality curves. Broken curves indicate the use of a lot of fish differing in resistance from the other lots used.

plotted parts of the survival-time and velocity-of-fatality curves obtained with rotenone and allied compounds. In the first p ace, this product is the constant of the equation of an equilateral hyperbola tangent to the survival-time curve at the point corresponding to the product. The curve on either side of this point is gradually compressed within the hyperbola. Thus, it is a simple means of obtaining a hyperbola approximating the critical portion of the curve; that is, it locates a point in that region of the curve where the inverse variation of survival time with variation in concentration is most nearly proportionate. In the second place, as shown in table 2, the product is that of the coordinates of the point corresponding to the condition of greatest efficiency with regard to the two variables, concentration and time; that is, the greatest speed of toxic action is attained with the smallest quantity of toxic substance. Beyond this point, acceleration of the speed of toxic action decreases with increase in concentration, as shown in the velocity-of-fatality curves in figure 2. Thus the point is one that is medianly located with respect to the preponderant influences of the concentration tolerance factor and the time tolerance factor.

TABLE 2.—*Correspondence of minimum ct with point of greatest efficiency*

Substance	Concentration corresponding to minimum *ct*	Concentration corresponding to point of greatest efficiency	Substance	Concentration corresponding to minimum *ct*	Concentration corresponding to point of greatest efficiency
	Milligram per liter	*Milligram per liter*		*Milligram per liter*	*Milligram per liter*
Rotenone	0.045	0.050	Tephrosin	0.15	0.11
Dihydrorotenone	.040	.045	Rotenone hydrochloride	.023	.022
Isorotenone	.14	.13	Acetylrotenone	.075	.075
Toxicarol	.075	.075	Rotenolone	.41	.40
Deguelin	.055	.045			

The nearer the arch is to the origin, the greater is the toxicity of the compound; therefore, the compounds, arranged according to their toxicities as represented by this portion of the graphs in figure 1, would have the following descending order: Rotenone hydrochloride, rotenone and dihydrorotenone, toxicarol, acetylrotenone, deguelin, isorotenone, tephrosin, and rotenolone. This order is given by the comparison of the minimum *ct* products as shown in tables 3 and 4 and by none of the other methods. In addition, the apparent grouping of the compounds (fig. 1) is obviously approximated by their respective minimum *ct* products, whereas, although some of the compounds may show the same order according to another method, the quantitative comparison in these cases is not that exhibited by the graphs.

In tables 3 and 4 are given the relative toxicity data used in a comparison of the methods considered. These data were taken from papers previously published by the author (*18*, *19*, *20*).

The values given in table 4 are the relative values of the toxicities of the substances as represented by the graphs. They are not the values for the true relative toxicities; to obtain these it would be necessary to correct some of the values given for any difference in

resistance of the different lots of fish, as shown by comparative tests with rotenone. This difference was significant only in the tests with rotenone hydrochloride, acetylrotenone, and rotenolone. That the curves may be separated into two actually comparable groups is indicated by the drawing of one in solid lines and the other in broken lines. The correction to be applied will depend on the method of determining the relative toxicity. If the minimum *ct* product is used as the criterion, the three substances mentioned must be referred to rotenone that has given a minimum *ct* of 9.5 with the same lot of fish. Dihydrorotenone was also tested again with this lot. The relative value obtained, 1.4, differed somewhat from that given previously, 1.1, but since it was obtained from a more carefully controlled series of tests, it should be used. Corrected in this way, the actual relative toxicities of the substances with respect to rotenone are as follows: Rotenone hydrochloride, 1.8; dihydrorotenone, 1.4; acetylrotenone, 0.55; toxicarol, 0.55; deguelin, 0.39; isorotenone, 0.23; tephrosin, 0.15; and rotenolone, 0.097.

TABLE 3.—*Toxicity data*

Substance	c_0	t_0	$\tan \theta$	$\sqrt{\tan \theta / c_0}$	$\sqrt{\tan \theta / c_0 t_0}$	Minimum *ct*	Minimum $(c-c_0)(t-t_0)$
	Milligram per liter	*Minutes*	*Liter per milligram per minute*		*Liter per milligram per minute*	*Milligram minutes per liter*	*Milligram minutes per liter*
Rotenone	0.012	40	0.19	4.0	0.58	7.7	4.4
Dihydrorotenone	.005	75	.16	5.6	.64	7.0	3.5
Isorotenone	.040	145	.042	1.0	.085	33	9.3
Toxicarol	.015	75	.097	2.5	.29	14	6.6
Deguelin	.030	80	.15	2.2	.25	20	6.8
Tephrosin	.055	135	.040	.85	.073	52	20
Rotenone HCl	.002	125	.22	10	.94	5.3	
Rotenolone	.020	115	.011	.74	.069	98	51
Acetylrotenone	.002	95	.067	5.7	.59	17	

TABLE 4.—*Relative toxicity as determined by different methods*

Substance	Ratio of slopes	Ratio of values by formula 1 [1]	Ratio of values by formula 2 [2]	Ratio of minimum *ct*	Ratio of minimum $(c-c_0)(t-t_0)$
Rotenone HCl	1.2	2.5	1.6	1.5	
Dihydrorotenone	.84	1.4	1.1	1.1	1.1
Rotenone	1.0	1.0	1.0	1.0	1.0
Toxicarol	.51	.63	.50	.55	.63
Acetylrotenone	.35	1.4	1.0	.43	
Deguelin	.79	.55	.43	.39	.56
Isorotenone	.28	.22	.14	.23	.53
Tephrosin	.23	.21	.13	.15	.20
Rotenolone	.058	.19	.12	.079	

[1] Powers' formula. [2] Suggested modification of Powers' formula.

SUMMARY

Of the equations examined, Glaser's equation, $(C-b)(1-e^{-a(t-\theta)})=k$, is the most nearly applicable to the concentration survival time curves obtained in studies with rotenone and related compounds in which the goldfish was the test animal. This equation, however, is too difficult to use in making a simple comparison of the toxicity of these compounds.

Powers' formula, toxicity $=\sqrt{\frac{\tan \Theta}{a}}$, is unsatisfactory. A modification of this formula which includes the time-tolerance factor, $\sqrt{\frac{\tan \Theta}{c_0 t_0}}$, is an improvement, but it, too, is unsatisfactory because of the necessity for determining the two tolerance factors.

The use of the slope of the theoretical velocity of fatality is unsatisfactory because it is difficult to locate accurately in some cases and because it is strongly influenced by the threshold value.

The minimum *ct* product is proposed as a criterion for comparing toxicities for the following reasons: (1) It always falls within that region of the curve in which the relation of concentration and survival time is most nearly rectangularly hyperbolic; (2) it corresponds to an intermediate condition between the predominant influence of the two tolerance factors; (3) it gives a value at the point of greatest efficiency with respect to concentration and time; and (4) it is comparatively easy to determine.

The minimum *ct* product when referred to the asymptotes as axes is unsatisfactory because of the difficulty of determining the asymptotes accurately.

The relative toxicities of the compounds studied, as determined by the minimum *ct* product, agree well with the apparent relation shown by the arching protions of their survival-time curves. This agreement is not shown by the other methods considered.

These values, referred to the value of rotenone for the same group of goldfish, are as follows: Rotenone hydrochloride, 1.8; dihydrorotenone, 1.4; acetylrotenone, 0.55; toxicarol, 0.55; deguelin, 0.39; isorotenone, 0.23; tephrosin, 0.15; and rotenolone, 0.097.

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THE QUANTITATIVE RELATIONSHIP BETWEEN THE CONSTITUTION AND TOXICITY OF SOME ROTENONE DERIVATIVES [1]

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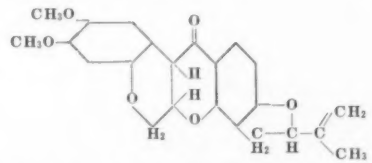
INTRODUCTION

The preparation [2] of a number of derivatives of rotenone incident to the determination of its chemical structure afforded an opportunity for studying the toxicity of various highly toxic substances differing from each other but slightly in constitution. The method of studying the toxicity of an individual substance, with the goldfish as the test animal, is described in a previous paper [3]. From the data obtained a survival-time curve in which concentration is plotted against survival time, and a velocity-of-fatality curve in which concentration is plotted against the reciprocal of the survival time are drawn for each of the various substances. From the latter curve a value may be obtained for the maximum rate of increase of the velocity of fatality with increase in concentration. Comparisons according to this criterion, which are more serviceable from the standpoint of practicality, inasmuch as they have reference to that portion of each curve in which the proportional change in the two variables, concentration and time, is not greatly different, permit the following generalizations to be made [4]: (1) The dihydro derivatives produced by saturation of the double ond in the side chain with hydrogen have appreciably higher toxicities b an the corresponding unsaturated compounds; (2) the enol acetates and the acetyl derivatives of the hydroxy compounds have appreciably lower toxicities than the parent compounds; and (3) the hydroxy derivatives have much lower toxicities than the parent compounds.

Eight of the compounds studied may be classified in the abovementioned groups—rotenone, dihydrorotenone, acetylrotenone [5], rotenolone [5], acetyldihydrorotenone, dihydrorotenolone, acetylrotenolone, and acetyldihydrorotenolone. The structure of rotenone [6] is as follows:



[1] Received for publication Feb. 18, 1935; issued July 1935. Presented before the division of biological chemistry at the meeting of the American Chemical Society held at St. Petersburg, Fla., Mar. 25 to 30, 1934.

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[6] LA FORGE, F. B., HALLER, H. L., and SMITH, L. E. See pp. 184, 191, and 202, respectively, of reference cited in footnote 2.

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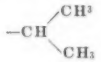
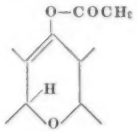
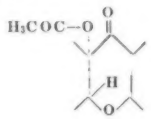
The dihydro derivatives are characterized by the saturation of the double bond in the side chain with hydrogen and so contain the group



The enol acetates, acetylrotenone and acetyldihydrorotenone, which are obtained [6] from rotenone and dihydrorotenone on treatment with sodium acetate and acetic anhydride, are characterized by the grouping



The acetyl derivatives of the hydroxy compounds, acetylrotenolone and acetyldihydrorotenolone, which are prepared [6] as the main products of the reactions of iodine and an alkali acetate with alcoholic solutions of rotenone and dihydrorotenone, respectively, are characterized by the grouping



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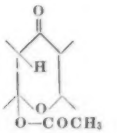

The hydroxy derivatives, rotenolone and dihydrorotenolone, are obtained [6] on saponification of the last-named acetyl compounds.

CRITERION FOR COMPARISON OF TOXICITY

Recently the author proposed [7] the use of the minimum product of concentration and survival time as a practical criterion for the comparison of toxicity. This value corresponds to a point at which neither tolerance factor is preponderant; it corresponds to the point of greatest efficiency with respect to concentration and survival time, and it is comparatively easy to determine.

The probable error of the observed *ct* products in the neighborhood of the minimum is no greater than 7 percent of the mean. However, the error of the given minimum value is even less, for it is determined from the survival-time curve, which is, in effect, an average of all the

[6] See footnote on page 893.

[7] GERSDORFF, W. A. A NEW CRITERION FOR THE COMPARISON OF TOXICITY WITH RESPECT TO CONCENTRATION AND TIME. Jour. Agr. Research. 50:881-891, illus. 1935.

observed points. The survival-time curves are given in figure 1. Since the points corresponding to the minimum *ct* products always appear on the arches of the curves, these portions only were determined in the comparative tests made with rotenone and dihydrorotenone.

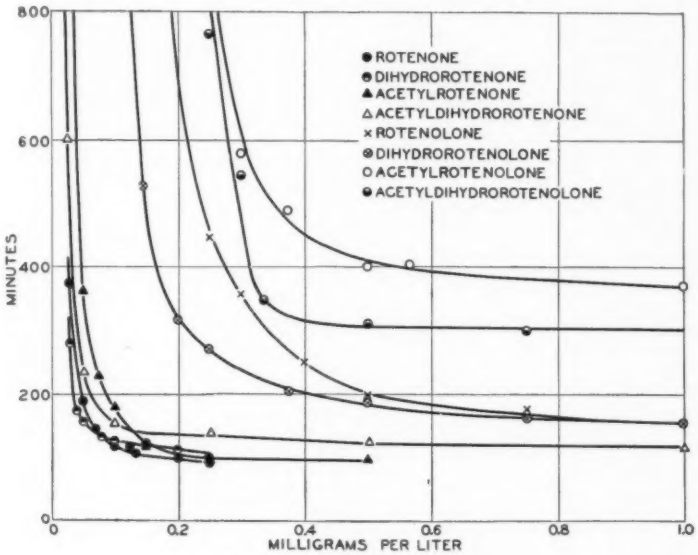

FIGURE 1.—Survival-time curves.

RESULTS

Since different lots of goldfish may vary somewhat in their resistance to toxic substances and two different lots were used in the toxicological tests described in this paper, check tests were made with each lot for the purpose of comparison, rotenone being used as the standard compound. The data for these tests are given in table 1.

TABLE 1.—*Toxicity of rotenone, dihydrorotenone, acetyldihydrorotenone, acetylrotenolone, dihydrorotenolone, and acetyldihydrorotenolone at 27.0° C. ± 0.2°*

ROTENONE

Concentration (mg per liter)	Fishes [1]	Mean length of fishes	Mean weight of fishes [2]	Mean survival time	Concentration (mg per liter)	Fishes [1]	Mean length of fishes	Mean weight of fishes [2]	Mean survival time
	Number	*Mm*	*Grams*	*Minutes*		*Number*	*Mm*	*Grams*	*Minutes*
0.200	[3] 6	43	2.4	115	0.084	5	44	2.5	138
0.200	9	42	2.3	118	0.070	8	43	2.4	146
0.132	4	43	2.4	121	0.052	10	45	2.6	185
0.124	9	43	2.4	128	0.050	[3] 7	46	2.7	190
0.100	19	44	2.5	123	0.027	[3] 13	47	3.1	375
0.100	[3] 7	44	2.5	130					

[1] Fishes from lot 4 were used in the dihydrorotenolone tests; fishes from lot 2 were used in the other tests, with the exceptions noted.
[2] Estimated from length exclusive of tail.
[3] From lot 4.

TABLE 1.—*Toxicity of rotenone, dihydrorotenone, acetyldihydrorotenone, acetylrotenolone, dihydrorotenolone, and acetyldihydrorotenolone at 27.0° C.±0.2°*—Contd.

DIHYDROROTENONE

Concentration (mg per liter)	Fishes	Mean length of fishes	Mean weight of fishes	Mean survival time	Concentration (mg per liter)	Fishes	Mean length of fishes	Mean weight of fishes	Mean survival time
	Number	*Mm*	*Grams*	*Minutes*		*Number*	*Mm*	*Grams*	*Minutes*
0.25	12	42	2.3	96	0.078	8	42	2.3	135
0.20	16	44	2.6	111	0.050	30	40	2.1	158
0.15	13	41	2.2	121	0.040	23	39	1.9	175
0.10	21	42	2.3	125	0.030	14	40	2.1	280
0.080	13	40	2.1	132					

ACETYLDIHYDROROTENONE

2.00	8	40	2.1	122	0.100	[3] 14	46	2.8	150
1.00	11	39	1.9	116	0.050	12	43	2.4	230
0.500	11	40	2.0	122	0.050	[3] 15	47	3.1	238
0.250	11	41	2.2	137	0.025	[3] 20	48	3.3	600
0.100	8	43	2.4	155					

ACETYLROTENOLONE

2.00	10	41	2.2	[4] 320	0.500	4	44	2.5	400
1.50	[3] 12	47	3.1	365	0.375	[3] 11	46	2.8	489
1.00	6	38	1.7	370	0.300	[3] 13	48	3.2	580
0.563	[3] 15	45	2.6	406	0.250	9	46	2.7	910

DIHYDROROTENOLONE

1.00	10	46	2.7	155	0.200	12	44	2.6	319
0.750	12	45	2.7	161	0.143	11	43	2.5	530
0.500	12	45	2.6	188	0.102	12	46	2.7	[5] 800
0.375	11	45	2.7	203	0.075	12	[6]	[6]	[7]
0.250	10	45	2.6	272					

ACETYLDIHYDROROTENOLONE

2.00	7	40	2.1	280	0.500	[3] 15	46	2.8	312
2.00	[3] 13	46	2.9	299	0.333	[3] 16	47	3.1	348
1.33	[3] 12	46	2.9	312	0.300	[3] 16	48	3.2	547
1.00	6	40	2.0	[8] 344	0.250	9	43	2.5	767
0.750	[3] 15	46	2.7	300	0.100	4	40	2.0	[9]
0.500	4	38	1.7	309					

[3] From lot 4.
[4] During the initial 15 minutes of the test the temperature was too high, being 27.7° C. at the start; therefore the survival time value recorded is probably somewhat low.
[5] Owing to an increase in room temperature overnight, the temperature of the test rose to 27.9° C.; therefore the value recorded for the survival time is probably low.
[6] All fishes were not measured, but they were of the same approximate size as in the other tests.
[7] 35 hours for 9 fishes; 3 fishes were apparently unaffected after 96 hours.
[8] The temperature was too low during this test, the average being only 26.0° C.±0.2°. Therefore, the value recorded for the survival time is high.
[9] 17 hours for 3 fishes; 1 fish was apparently unaffected after 48 hours.

In the tests on three of the compounds both lots [8] were used. In the tests with dihydrorotenolone lot 4 alone was used. This lot was chosen to finish the tests because the goldfish, although of larger average size, were apparently similar to lot 2 in their resistance to these toxic substances. The results obtained with them fall well within the limit of error of the tests with lot 2. This fact is shown by the tests with all the compounds in which both lots were used. Lot 2 is from the same group of fish that was used in the previously pub-

[8] Lot 2 was obtained in October 1931, and lot 4 was obtained in January 1933.

lished tests with rotenolone and acetylrotenone.[9] The toxicity data for the derivatives other than these two are given in table 1.

The relative toxicity of the derivatives, obtained by comparison of their respective minimum *ct* products, designated $(ct)_m$, with that of rotenone is given in table 2. In table 3 the relation between the structure of the compounds and their toxicity is shown by the ratios between the values for the parent compounds and their derivatives. They are arranged according to the three groups, the dihydro derivatives, the acetates, and the hydroxy derivatives. There are four ratios in each group, and it is apparent that within each group they are practically identical.

TABLE 2.—*Relative toxicity of rotenone derivatives at 27.0° C. ± 0.2°*

Compound	Concentration	Survival time	Minimum *ct*	Relative toxicity referred to rotenone
	Milligram per liter	*Minutes*	*Milligram minutes per liter*	
Dihydrorotenone	0.035	195	6.83	1.39
Rotenone	.050	190	9.50	1.00
Acetyldihydrorotenone	.050	234	11.7	.812
Acetylrotenone	.075	229	17.2	.552
Dihydrorotenolone	.200	319	63.8	.149
Rotenolone	.410	240	98.4	.0965
Acetyldihydrorotenolone	.330	352	116	.0819
Acetylrotenolone	.370	470	174	.0546

TABLE 3.—*The effect of slight changes in the consitution of rotenone compounds on their toxicity*

Compound	$(ct)_m$	Effect of H[1]	Effect of Ac[2]	Effect of OH[3]	Effect of H and Ac	Effect of H and OH	Effect of Ac and OH	Effect of H, Ac, and OH
	Milligram minutes per liter	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Rotenone	9.50							
Dihydrorotenone	6.83	139						
Acetylrotenone	17.2		55.2					
Acetyldihydrorotenone	11.7	147	58.3		81.2			
Rotenolone	98.4			9.7				
Dihydrorotenolone	63.8	154		10.7		14.9		
Acetylrotenolone	174		56.6	9.9			5.46	
Acetyldihydrorotenolone	116	150	55.0	10.1	84.8	14.8	5.89	8.19
Average		148	56.3	10.1	83.0	14.9	5.68	
Product of individual effects					83.3	14.9	5.69	8.41

[1] 100 times the ratio of the $(ct)_m$ of the unsaturated parent compound to that of the dihydro derivative.
[2] 100 times the ratio of the $(ct)_m$ of the parent compound to that of the acetate.
[3] 100 times the ratio of the $(ct)_m$ of the parent compound to that of the hydroxy derivative.

SUMMARY AND CONCLUSIONS

Eight compounds, rotenone and seven of its derivatives, compared according to toxicity under conditions corresponding to the minimum value of the *ct* product at 27° C., have the following relative toxicities

[9] GERSDORFF, W. A. See footnote 5.

to goldfish weighing from 2 to 3 g. Dihydrorotenone, 1.4; rotenone, 1.0; acetyldihydrorotenone, 0.81; acetylrotenone, 0.55; dihydrorotenolone, 0.15; rotenolone, 0.097; acetyldihydrorotenolone, 0.082; and acetylrotenolone, 0.055.

Insofar as these compounds and this method of comparison are concerned, each change in chemical constitution effects a characteristic change in toxicity independent of the effect of any other change.

The dihydro derivatives produced by saturation of the double bond in the side chain with hydrogen have 1.5 times the toxicity of the corresponding unsaturated compounds.

The acetates, whether of the enol type or the acetyl derivatives of the hydroxy compounds, have 0.56 the toxicity of the parent compounds.

The hydroxy derivatives have 0.10 the toxicity of the parent compounds.

The combined effect on toxicity of more than one change in constitution is equal to the product of the individual effects. Thus, the dihydroacetates have 0.83 the toxicity of the parent compounds; the dihydrohydroxy derivatives have 0.15 the toxicity of the parent compounds; the acetylhydroxy derivatives have 0.057 the toxicity of the parent compounds; and acetyldihydrorotenolone, the derivative including all three changes in constitution, has 0.082 the toxicity of rotenone.

EFFECT OF GOSSYPOL UPON THE MUSCLE OF THE SMALL INTESTINE OF THE RAT[1]

By L. A. MOORE[2]

Research assistant in dairy husbandry, Michigan Agricultural Experiment Station

INTRODUCTION

Although gossypol, the toxic principle of cottonseed, has been widely studied, its physiological effect on the muscle tissue of the intestinal tract appears to have received little attention. Schwartze and Alsberg (*8, p. 194*)[3] stated, "Gossypol has a paralytic effect not merely upon the neuromuscular apparatus of striated muscle but also upon that of smooth muscle", and in another article (*7, p. 176*) they reported, "The oral administration of gossypol dissolved in oil is followed by diarrhea and also by paralysis of the intestine and stomach musculature."

Edgerton and Morris (*3*) noted in the post-mortem examination of four rabbits which died of cottonseed poisoning that the stomach contained a large amount of food mixed with mucus and that the intestines were full. Macy and Mendel (*5*) made a similar observation on rabbits fed cottonseed meal. Gallup (*4*) noticed intestinal impaction in a rat which lived 8 days after an intraperitoneal injection of gossypol. Clark (*2*) found intestinal impaction in the chronic type of poisoning where rats were injected intraperitoneally. Macy and Outhouse (*6*) noticed that dogs fed a diet containing 45 percent of cottonseed meal for 192 days showed extreme soreness in the abdominal regions. Withers (*9*) noted pendulous crops in hens receiving 0.2 percent of gossypol in the diet. Thirty percent of raw cottonseed produced the same effect.

The literature cited indicates that gossypol has a paralytic effect upon the muscle tissue of the digestive tract of animals. Unpublished work by the author showed that gossypol, in oil solution, given in sufficient amounts by means of a stomach tube, caused a paralysis of the stomach musculature, and that when added directly to the bathing solution it caused paralysis of the muscle of the isolated uterus of the rat. The present work was carried out to determine the effects of gossypol upon the isolated longitudinal muscle of the small intestine of the rat.

PROCEDURE

The principal method of study was to observe the contractility of longitudinal strips of intestine after the rats had been fed gossypol or raw crushed cottonseed in various amounts for different lengths of time, the usual technic for such tissues being used. It was thought that such a procedure would more closely record the effects of gossypol than a study of the effects of gossypol added directly to the isolated

[1] Received for publication Jan. 15, 1935; issued July 1935. Journal article no. 208 (n. s.) of the Michigan Agricultural Experiment Station.

[2] The author wishes to express his appreciation to Prof. B. B. Roseboom, of the physiology department of Michigan State College, for his interest in this work and for the use of apparatus in carrying out these experiments.

[3] Reference is made by number (italic) to Literature Cited, p. 908.

strips of intestine. The latter method, however, was used in a few experiments, with concentrations of gossypol at 1 to 6,000, 1 to 12,500, and 1 to 25,000.

The gossypol used was prepared in the form of an acetate by Carruth's method (*1*). The acetic acid was removed by dissolving the gossypol acetate in ether and washing with water. The ether was driven off on a steam bath at 60° C. The crusts formed were washed with water and recrystallized from ether.

Albino and piebald rats from a healthy colony which received a ration conducive to good growth and reproduction were used. The experimental rats were generally started on the gossypol or raw crushed cottonseed diets at 30 to 40 days of age. Litter mates of the same sex and approximately of the same weight were used as controls. The experimental animal of each pair received a diet containing gossypol or raw crushed cottonseed. The control animal received the same amount of food per day without the gossypol or raw cottonseed as the experimental animal had consumed the previous day. It was thought best to follow this procedure in order to eliminate any effects which the lowered food consumption might have on the experimental animals. Except for the effect of the gossypol, the control animals would approximate the weight and nutritive state of the experimental animals at the time of slaughter. All animals had access to food up to the time they were killed.

The following basal diet was used:

	Percent
Ground yellow corn	54
Wheat middlings	23
Refined cottonseed oil	7
Alfalfa leaf meal	4
Protein mixture	9
Calcium carbonate	1
Salt	1
Bonemeal	1
	100

The protein mixture contained:

Tankage	50
Linseed meal	35
Crude casein	15
	100

When gossypol was added to the diet, the desired amount was dissolved in ether and mixed with the cottonseed oil used in the ration. This solution was poured over the dry feed mixture, which was then spread in a thin layer and allowed to remain at room temperature until the ether had evaporated. Where raw crushed cottonseed was fed it replaced the wheat middlings in the basal diet.

After the animals had been on the diets the required length of time they were removed from the cages and killed by a blow on the head. The abdomen was opened and strips about 2 cm in length were removed and placed in Locke's solution without glucose. The strips were taken from three different portions of the intestine. Most of them were taken from 12 to 25 cm below the pylorus. Others were taken from 3 to 5 cm below the pylorus and from 3 to 5 cm above the caecum. The strips were removed as needed from Locke's solution without glucose, and kymograph records were made of

their longitudinal activity in the complete Locke's solution. Oxygen was bubbled through the Locke's solution with the temperature kept constant at 37.5° C. Contractility records were made simultaneously on segments from the control and experimental animals.

The levers used measured 19 cm from the writing point to the fulcrum and 3.5 cm from the fulcrum to the thread attached to the intestine, and were so weighted as to place a stress of about 0.5 g on the strip.

In order to simplify the description and recording of the results of the contractility studies a grading system was used. Four plus means that a strip had consistently made excursions in height of contraction comparable with that which a beating strip would make

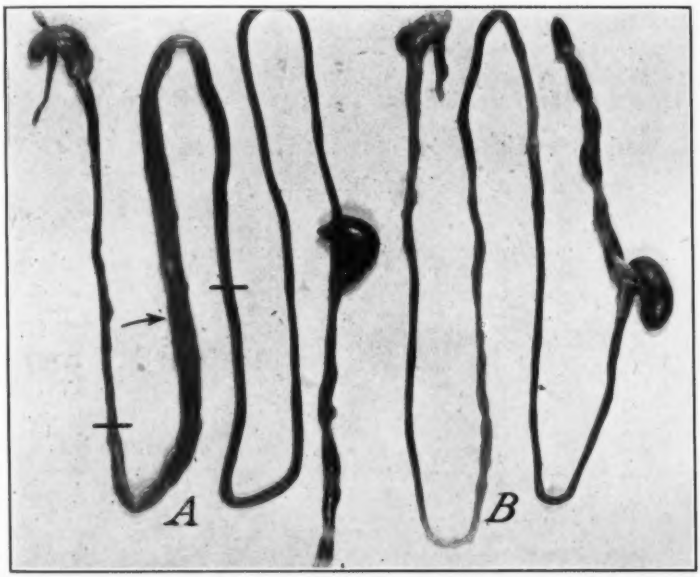

FIGURE 1. —Dilated intestine of a rat fed 17 percent of raw cottonseed for 8 weeks (*A*) compared with that of the control animal fed the same kind and amount of food without the raw cottonseed (*B*). The dilation was graded +++ to ++++.

which was normal for the weight of the animal and the particular place in the intestine from which it was taken. Zero means that the strip showed little or no activity; 1+, 2+, and 3+ represent intermediate values, and 5+ or 6+ means that the height of the excursions was greater than normal.

During the progress of the experiments it was noticed that many of the experimental animals which had received gossypol or raw cottonseed for 3 to 8 weeks in amounts that would limit growth, showed a dilation of a part of the small intestine, as indicated in figure 1. These distended or dilated intestines were graded as +, ++, +++, and ++++ according to the severity of the distension.

It was noticed during the progress of the experiments that strips of intestines from the experimental animals when placed in Locke's

solution without glucose did not shorten as much as similar strips taken from the control animals. Accordingly 6 cm strips were taken 12 to 25 cm below the pylorus from each pair of animals and placed in Locke's solution without glucose. Approximately 1 hour later they were removed, measured again, and the length recorded. Inasmuch as the strips from the various pairs were kept at different temperatures, only the strips from each pair are comparable.

Kymograph records were not made of pairs in which the experimental animals appeared to be within a few days of death.

In the tables the letter E before the numbers of an animal designates the experimental animal and the letter C designates the control.

EXPERIMENTAL RESULTS

EFFECT OF ADDING GOSSYPOL DIRECTLY TO THE SOLUTION BATHING A NORMAL INTESTINE

To determine the effect of adding gossypol directly to a solution bathing a normal intestine, two strips of intestine were taken from the same animal 12 to 25 cm below the pylorus, and one was placed in a solution to which gossypol was added at a concentration of 1 to

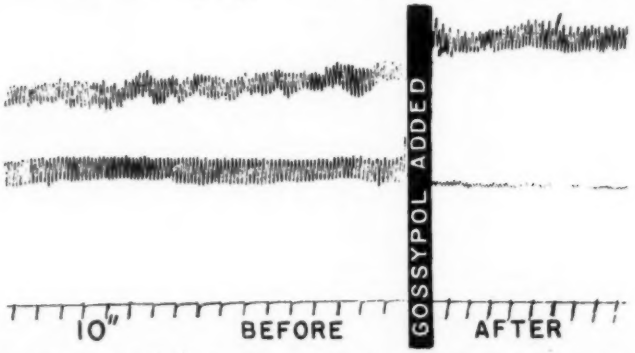

FIGURE 2.—Effect on isolated longitudinal strips of the intestine of a rat before, and 13 minutes after, adding gossypol directly to the bathing solution at a concentration of 1 to 6,000. 10" = 10 seconds, time interval.

6,000. The other strip served as a control. The results, presented as kymograph records in figure 2, show that at a concentration of 1 to 6,000 the contractions were greatly reduced after 13 minutes. At a concentration of 1 to 25,000, as shown by other experiments, the contractions were greatly reduced after 20 minutes.

Apparently gossypol when added directly to the bathing solution caused a progressive paralysis of the muscle of the small intestine.

EFFECT OF FEEDING FROM 0.2 TO 0.4 PERCENT OF GOSSYPOL FOR 5 TO 21 DAYS ON SEGMENTS OF INTESTINE TAKEN FROM 12 TO 25 CM BELOW THE PYLORUS

By reference to table 1 it will be noted that three animals fed 0.2 percent gossypol for 17 days showed normal contraction. In these cases, however, the 6-cm strip did not shorten as much as that of the controls. In none of these three animals was the intestine dilated.

TABLE 1.—*Effect of feeding rats from 0.2 to 0.4 percent of gossypol, from 5 to 21 days, upon the contractile strength of strips of their intestines taken from 12 to 25 cm below the pylorus*

Animal no. and sex [1]	Weight when placed on experiment	Weight when killed	Gossypol fed	Feeding period	Dilation of intestine [2]	Strips used	Length of 6-cm strip 1 hour after removal	Strength of contraction
	Grams	*Grams*	*Percent*	*Days*		*Number*		*Centimeters*
E 541, male	71	88	0.2	17	0	2	4.0	4+
C 540, male	72	86			0	2	3.5	4+
E 547, male	83	91	.2	17	0	2	4.2	4+
C 546, male	92	97			0	2	4.0	3+
E 553, male	101	105	.2	17	0	2	3.5	4+
C 552, male	101	97			0	2	3.4	4+
E 443, male	63	60	.3	5	0	1	3.5	6+
C 444, male	64	55			0	1	3.3	5+
E 437, male	64	55	.3	7	0	2	3.4	6+
C 438, male	69	65			0	2	3.0	5+
E 439, female	102	81	.3	14	++	2	4.1	6+
C 440, female	103	92			0	2	3.3	3+
E 441, female	58	51	.3	14	+	2	4.3	6+
C 442, female	67	52			0	2	3.3	4+
E 448, male	66	49	.3	18	++	2	4.0	5+
C 447, male	65	67			0	2	3.5	4+
E 418, female	62	73	.2 .25 .3	7 7 7	++++	1	4.4	5+
C 417, female	58	69			0	1	3.4	4+
E 480, male	61	43	.4	7	0	2	4.2	6+
C 479, male	61	49			0	2	3.5	4+
E 484, female	64	46	.4	8	0	2	4.5	6+
C 483, female	63	54			0	2	3.3	4+
E 486, female	45	45	.4	10	0	2	3.4	5+
C 485, female	65	40			0	2	2.8	3+
E 488, female	69	39	.4	9	Died			
C 487, female	67	49						
E 490, male	56	38	.4	11	Died			
C 489, male	56	35						
E 482, female	61	38	.4	12	Died			
C 481, female	61	36						

[1] E, experimental, and C, control animals in all tables.
[2] See text for explanation of symbols in this and following tables.

Of five animals fed 0.3 percent of gossypol for from 5 to 18 days, all showed an increased height of contraction as compared with the controls. In all cases the 6-cm strip from the experimental animal shortened less than normal. The tracings of experimental animal E 439 and control animal C 440 are presented in figure 3.

One animal (E 418) fed 0.2 percent of gossypol for 7 days, 0.25 percent for 7 days, and then 0.3 percent for 7 days more showed an increased height of contraction as compared to the control. This animal showed a ++++ distension of the intestine.

Three animals fed 0.4 percent of gossypol for 7 to 10 days showed increased contraction as compared to the controls. Three other animals at this level of gossypol died in from 9 to 12 days.

It would appear from this series of observations that gossypol fed at levels of 0.3 to 0.4 percent for short periods causes an increase in the height of contraction of longitudinal strips of the intestine. The 6-cm strips, however, shortened less than those of the controls and four animals of the group showed a distended intestine.

EFFECT OF FEEDING FROM 0.2 TO 0.3 PERCENT OF GOSSYPOL FOR 21 TO 42 DAYS

A series of experiments was carried out to determine the effects of feeding 0.2 to 0.3 percent of gossypol for 21 to 42 days. The tracing in figure 4, shows an almost complete paralysis of the segment of intestine of rat E 502, after the animal had received 0.3 percent of gossypol for 2 weeks, 0.2 percent for 2 weeks, and 0.25 percent for 3

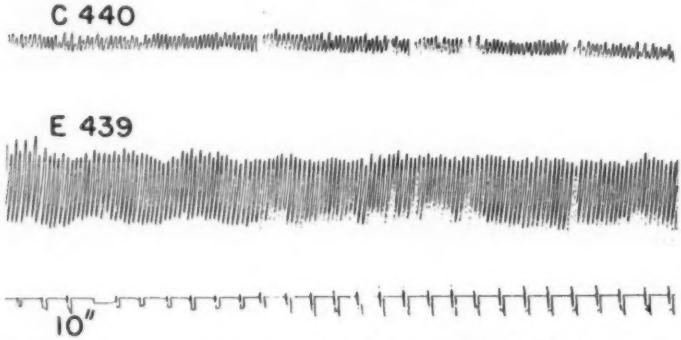

FIGURE 3.—Showing increased activity of longitudinal strip of intestine of rat E 439 fed 0.3 percent gossypol for 14 days compared to control animal C 440. 10" = 10 seconds time interval

weeks. This result was characteristic of those obtained in this series of experiments. The 6-cm strip of the intestines of the experimental animals contracted but very little as compared with that of the controls (table 2).

An experiment in which five animals were fed 0.15 percent of gossypol for 10 days and 0.3 percent for 32 days, but in which the food consumption of the controls was not limited, gave the same results.

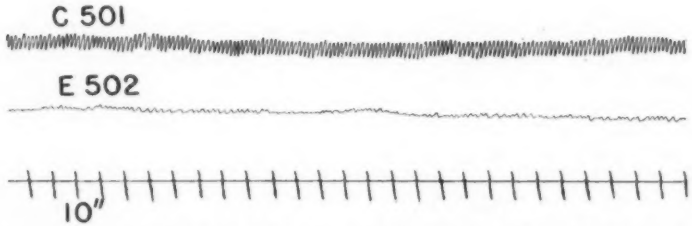

FIGURE 4.—Almost complete paralysis of the segment of intestine of rat E 502 fed 0.3 percent gossypol for 2 weeks, 0.2 percent for 2 weeks, and 0.25 percent for 3 weeks as compared with control animal C 501. 10" = 10 seconds time interval

A comparison of the results shown in table 1 with those in table 2 suggests that a large amount of gossypol fed for a short period causes a stimulation of the longitudinal strips and that the feeding of smaller amounts for longer periods finally causes a paralysis of the strips.

TABLE 2.—*Effect of feeding rats from 0.2 to 0.3 percent gossypol, from 21 to 42 days, upon the contractile strength of strips of their intestines taken from 12 to 25 cm below the pylorus*

Animal no. and sex	Weight when placed on experiment	Weight when killed	Gossypol fed	Feeding period	Dilation of intestine	Strips used	Length of 6-cm segment 1 hour after removal	Strength of contraction
	Grams	*Grams*	*Percent*	*Days*		*Number*	*Centimeters*	
E 504, female	80	58	0.3 .25	7 35	+	1	5.5	1+
C 503, female	75	72			0	1	3.5	3+
E 506, male	83	61	.3 .25	7 35	+	1	5.5	1+
C 505, male	73	76			0	1	3.5	4+
E 510, female	83	70	.3 .25	7 35	0	1	5.5	1+
C 509, female	75	91			0	1	3.5	4+
E 508, male	73	58	.3 .25	7 35	++++	------	[1]	------
C 507, male	73	95						
E 498, male	100	106	.3 .2 .25	14 14 12	+	1	5.7	2+
C 497, male	88	175			0	1	3.0	3+
E 500, male	88	79	.3 .2 .25	14 14 12	++++	1	5.5	1+
C 499, male	86	100			0	1	3.1	4+
E 502, male	87	92	.3 .2 .25	14 14 12	+	1	5.5	1+
C 501, male	87	100			0	1	3.7	4+
E 408, male	62	71	.2 .25 .3	7 7 7	+++	2	5.1	1+
C 407, male	64	74			0	2	3.0	4+
E 416, female	73	63	.2 .25 .3	7 7 4	++	2	4.8	1+
C 415, female	67	71			0	2	3.0	4+

[1] Almost dead.

EFFECT OF FEEDING 17 PERCENT OF RAW COTTONSEED FOR 42 TO 56 DAYS ON SEGMENTS OF INTESTINE TAKEN FROM 12 TO 25 CM BELOW THE PYLORUS

Although the experiments thus far reported were carried out with gossypol directly, in most of the preliminary work raw cottonseed was used as the source of gossypol. The results with three pairs of animals are shown in table 3. The effects were much the same as when gossypol was fed.

TABLE 3.—*Effect of feeding rats 17 percent raw cottonseed, from 42 to 56 days, upon the contractile strength of strips of their intestines taken from 12 to 25 cm below the pylorus*

Animal no. and sex	Weight when placed on experiment	Weight when killed	Feeding period	Dilation of intestine	Strips used	Length of 6-cm segment 1 hour after removal	Strength of contraction
	Grams	*Grams*	*Days*		*Number*	*Centimeters*	
E 512, male	71	80	42	++	2	5.6	0
C 511, male	75	100	------	0	2	3.5	4+
E 516, female	80	75	42	+++	2	5.3	2+
C 515, female	74	85	------	0	2	3.5	3+
E 514, male	71	100	56	+	1	5.8	0
C 513, male	65	117	------	0	1	3.7	3+

In another series of experiments four animals fed 17 percent of raw cottonseed for 5 weeks were killed and the segments from three different portions of the small intestine were compared directly with each other. The two segments taken from the upper part of the digestive tract showed marked paralysis, while those taken from 3 to 5 cm above the caecum were but slightly affected.

TABLE 4.—*Comparative effect of feeding gossypol to rats upon the contractile strength of strips from different parts of their intestines*

Animal no. and sex	Strength of segment 3–5 cm below the pylorus	Strength of segment 12–25 cm below the pylorus[1]	Strength of segment 3–5 cm above the caecum
E 504, female	0	1+	3+
C 503, female	2+	3+	1+
E 506, male	0	1+	4+
C 505, male	4+	4+	4+
E 510, female	1+	1+	3+
C 509, female	2+	4+	3+
E 500, male	0	1+	2+
C 499, male	4+	4+	3+
E 502, male	0	1+	4+
C 501, male	4+	4+	3+

[1] Data taken from table 2.

EFFECT OF GOSSYPOL ON SEGMENTS FROM OTHER REGIONS OF THE SMALL INTESTINE

In the experiments thus far presented all the segments studied were taken from 12 to 25 cm below the pylorus. This region was selected because it was here that the effect of the gossypol seemed to be most pronounced as judged by the fact that at this place a dilation of the intestine usually occurred.

From animals E 504, E 506, E 510, E 500, and E 502 (table 2) two strips were taken, one from 3 to 5 cm below the pylorus and the other from 3 to 5 cm above the caecum, and compared with similar strips from the controls. The results are shown in table 4.

PECULIAR RHYTHM IN DILATED SEGMENTS

A peculiar rhythm was noticed in eight segments from intestines which were greatly dilated—a number sufficiently large to be of some significance. This rhythm is shown in figure 5. The length of time between these peculiar reactions as well as the strength of contraction varied, of course, with different animals. This rhythm was never noticed except in the dilated portions of the intestine. In three instances it was duplicated in a second strip from the same animal.

DISCUSSION OF RESULTS

To conserve space, only the results from typical series of animals have been reported. The results of the investigation indicate that when gossypol is fed to rats in large amounts for 5 to 21 days, it causes a stimulation of the isolated longitudinal muscle of the small intestine, as shown by a study of strips taken from 12 to 25 cm below the pylorus. This effect may in part explain why investigators in the past have noticed diarrhea after gossypol or raw cottonseed has been fed to

animals. It may be that the increased stimulation of the intestine was an attempt of the animal to get rid of the toxic material. This stimulation was not apparent when gossypol was added directly to the isolated tissue in the bathing solution. It would seem, therefore, that the method used in this work more closely records the true physiological effects of a drug than the method of adding the material directly to the bathing solution.

The results further indicate that when the animals survive a medium dosage of gossypol for 4 to 8 weeks, a paralysis of the isolated longitudinal muscle takes place in the region about 12 to 25 cm below the pylorus. These animals would be considered by most investigators as in a chronic state of injury. No explanation of the mechanism by which the paralysis is produced will be attempted. A considerable number of segments of the various animals were subjected to pilocarpine in dilutions of 1 to 50,000. The results, however, were not consistent except in cases where the strips showed almost complete paralysis. In these cases the strips did not react to pilocarpine.

Since large amounts of gossypol fed for short periods stimulated the contractility of the muscle of the intestine in strips taken from 12 to

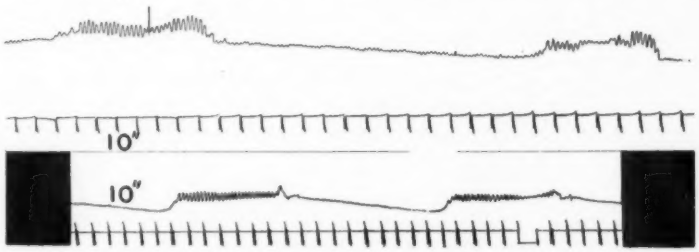

FIGURE 5.—Peculiar rhythm in strip of intestine taken from two different rats with a dilated segment of intestine. 10" = 10 seconds time interval.

25 cm below the pylorus and small amounts fed for long periods produced paralysis, it might be expected that the feeding of intermediate amounts for intermediate lengths of time would produce results in which some strips would show stimulation, some paralysis, and some would appear normal. This was the case in several series of experiments not reported in this paper.

Observations on different portions of the small intestine indicated that the lower portion of the small intestine was not markedly affected in animals in a chronic state of injury. Segments taken from 3 to 5 cm above the caecum contracted in about a normal manner.

During the progress of this experiment it was noticed that strips of intestine from animals fed gossypol became fatigued more easily than those from the controls. When animals had been fed gossypol for a considerable length of time, and records were made of strips taken from the same part of the intestine, it was found that if the first strip showed some activity, the second showed less and the third still less. In the case of the controls, however, the records showed no such decrease in activity.

Dilation of the intestine occurred in 22 of the animals reported in this investigation and in 33 of those not reported. The dilation could

be produced either with gossypol or raw cottonseed with equal regularity. It occurred most frequently in the animals that showed slow growth during the time they received the gossypol or raw cottonseed. It always occurred in the region indicated by an arrow in figure 1. In some cases the dilated segment of intestine was 7 or 8 times the normal size. Histological sections of the enlarged portion did not show any particular alteration.

In most cases the strips of intestine from the animals fed gossypol lacked the ability to shorten, although in certain instances they displayed some contractility. This observation is of some importance in this problem, for it seems to indicate that the musculature of the intestine had been affected in some way.

Although no kymograph records were made of the circular muscle of the small intestine, it might be expected that this muscle would be affected in the same manner as the longitudinal muscle since histologically it is the same type of muscle. The fact that segments of the intestines of the animals fed gossypol were dilated lends support to this view.

The evidence indicates that gossypol has a definite effect on the neuromuscular system of the upper portion of the small intestine of the rat, as is shown by the lowered strength of contraction, dilation of that portion of the intestine, and lack of ability of the segments to shorten.

It cannot definitely be stated whether the absence of effect on the lower portion is due to the fact that the gossypol has been digested and detoxified in the upper portion of the intestine and therefore does not have a chance to act on the lower portion, or whether gossypol acts specifically on certain portions of the tract.

No explanation is offered for the peculiar rhythm noticed in a few of the intestines which were greatly dilated. This type of contraction was never observed in any part of the intestine except the dilated part.

SUMMARY

Gossypol fed to rats at a 0.3- to 0.4-percent level for 5 to 21 days caused a stimulation of the isolated longitudinal muscle of the small intestine in segments taken from 12 to 25 cm below the pylorus.

Gossypol fed to rats at a 0.2- to 0.3-percent level for periods greater than 3 weeks and up to 8 weeks caused a partial paralysis of the isolated longitudinal muscle of the small intestine in segments taken from 12 to 25 cm below the pylorus.

In a limited number of rats in a state of chronic injury segments taken from 3 to 5 cm above the caecum were not markedly affected.

Gossypol fed at levels that permitted little growth usually caused a dilation in the upper portion of the small intestine of the rat.

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273—35——4

A STUDY OF THE PALATABILITY AND POSSIBLE TOXICITY OF 11 SPECIES OF CROTALARIA, ESPECIALLY OF C. SPECTABILIS ROTH [1]

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INTRODUCTION

Various species of the genus *Crotalaria* have been found to be adapted culturally to the lighter sandy soils of the Coastal Plain (*5*) [3] and are comparable in composition at early stages of growth with other legumes used in feeding livestock. Since within the genus, certain species perhaps have value as feeds for livestock, a joint study was undertaken by the United States Department of Agriculture and the Florida Agricultural Experiment Station, to locate and investigate such promising species. An investigation was undertaken to compare, by means of grazing and feeding trials, the relative palatability of the green forage, the artificially dried hays, and the silages made from several species. Tests were made in 1931, 1932, and 1933. Indications of the toxicity of one species—*C. spectabilis* Roth—to cattle were observed among the animals used, as was stated in a previous publication (*7, footnote, p. 621*). In December 1931 Thomas encountered field cases of toxicity of the seeds of this species to chickens, and studied more closely in the laboratory the reactions of chickens, turkeys, quail, and doves to these seeds (*9*). The toxic principle was isolated by Neal and Rusoff (*6*), who described it and studied certain of its properties. Emmel, Sanders, and Henley (*4*) recently found ground seeds of *C. spectabilis* to be toxic to swine.

Four species of *Crotalaria* are known to be toxic to livestock. Symptoms and lesions differ, indicating that the toxic principles are not the same in all species. Bessey and Stalker (*2*) showed *C. sagittallis* L. to cause "Missouri River bottom disease", which resulted in death among horses. Seeds of *C. juncea* L., or sunn-hemp (*1*) were fed to a healthy mature sheep in amounts of one-fourth pound daily for 14 days, and one-half pound for 12 additional days. Weakness, a tucked-up appearance, and catarrh developed shortly after the fourteenth day. Death occurred on the twenty-sixth day. According to Burtt-Davy (*3*), cattle fed *C. burkeana* Benth for 5 days became stiff in the joints, moved slowly, and were unable to stand ultimately. The hoofs lengthened and broke, and death resulted from starvation.

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[1] Received for publication Feb. 11, 1935; issued July.

[2] This study is an outgrowth of a cooperative investigation of the feeding value of crotalarias conducted jointly by the Division of Forage Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Departments of Agronomy and Animal Husbandry, Florida Agricultural Experiment Station. Feeding trials were conducted by the Department of Animal Husbandry, with forages grown and provided by the Agronomy Department and by the Division of Forage Crops and Diseases, U. S. Department of Agriculture. G. E. Ritchey was in charge locally for the Division of Forage Crops and Diseases.

[3] Reference is made by number (italic) to Literature Cited, p. 922.

Theiler (*8*) fed as much as 46 pounds of *C. dura* Wood and Evans to horses in 23 days and observed symptoms of poisoning within 16 to 80 days. The main symptoms and lesions included fever, acute polypnoea, and subsequent dyspnea, destruction of the respiratory epithelium, leading to a vicarious emphysema, granulation tissue, and degeneration of the bronchii. A bull fed 2 pounds daily died in 64 days, and an ox was killed in extremis on the ninety-eighth day. Symptoms included complete loss of appetite, diarrhea with dark-colored feces, progressive weakness, and ultimate loss of animation. Autopsy revealed cirrhosis of the liver, thickened central veins, and fibrillar bundles which formed a lacework in which blood collected.

Crotalaria striata D. C. and *C. incana* L. have been indicated as possibly toxic to cattle, sheep, and goats in certain countries, but have not been observed to be injurious in the United States.

PLAN OF INVESTIGATION

Nine species of *Crotalaria* were planted in adjacent rows in a fenced 2-acre field in 1931 and in 1932. From time to time during the grazing season, two head of cattle were transferred to this field for a brief tour, usually 14 days and the extent of grazing on the separate species was estimated daily by two or more members of the staff. No supplementary feeds were allowed. Water was accessible. Also, some volunteer grasses and other plants, not killed by routine cultivation of the field, were grazed.

Eight of the eleven species of *Crotalaria*, as listed in table 1, were harvested, artificially dried as hay, and placed in adjacent compartments of an overhead rack in a small lot, where cattle were given free access to them. Observations were made daily by two or more staff members as to the order of preference and relative amounts of each species eaten by the cattle. These observations, as well as the growth habits of the plants, were used as a basis for selection of the most promising species for further study as a forage crop. During the course of these studies, indications of toxicity of one species were obtained upon the death of three experimental animals. An additional controlled-feeding trial and careful laboratory analyses were made to prove the definite toxicity of the species *C. spectabilis*. Pertinent details relative to the toxicity of this species are outlined in this paper.

OBSERVATIONS OF CATTLE

Two cows were used in each tour on the crotalaria grazing plots in 1931 and 1932, the several species employed being listed in table 1. Four cows and two pigs were employed in this manner during the first season. The pigs grazed none of the *Crotalaria* during the 15-day tour. During the last 4 days of the fourth tour (Sept. 25–28, 1931), when *C. spectabilis* was in the early bloom, two cows stripped a considerable number of green leaves from the plants, but ate none of the stems nor flowers. They were returned to the dairy herd at the end of the 2-week tour, as originally planned. No outward symptoms of ill effects were noted immediately, nor later, when again under daily observation on the hay trials. On the basis of these earlier observations, none of the plants under trial were suspected of being toxic.

TABLE 1.—*Species of the genus Crotalaria*[1] *used in palatability trials*

Species	Class of roughage offered					
	Green forage		Artificially dried hay		Silage	
	1931	1932	1931	1932	1932	1933
C. anagyroides H. B. K.		×		×		×
C. goreensis Guill. and Perr.						×
C. grantiana Harvey	×	×	×	×		×
C. incana L.	×	×	×	×	×	
C. intermedia Kotschy	×	×	×	×	×	×
C. lanceolata E. Mey.	×	×	×	×		××
C. maxillaris Klotzsch	×	×				
C. retusa L.	×					
C. spectabilis Roth	×	×	×	×	×	
C. striata D C.	×	×	×	×	×	
C. usaramoensis Baker	×	×	×	×		×

[1] × signifies use of the plant in the manner indicated; ×× signifies use of the plant twice.

Three of the cows mentioned above, and 11 heifers, were used shortly afterward in 1 or more of a series of 8 palatability trials in which artificially dried hay was used. *Crotalaria spectabilis* hay was eaten by the cows in only one of the trials mentioned above, the amount being 12 pounds. No untoward effects were noted from this amount. It may be mentioned that the cows weighed between 900 and 1,000 pounds, so that the intake per 1,000 pounds live weight was relatively small. In these palatability trials, *C. spectabilis* ranked at the bottom of a group of eight species of crotalaria hays. These observations ranged from 14 to 38 cow-days per individual animal on the grazing trials, and from 17 to 24 days per individual on the hay trials during the first year.

These palatability trials were repeated in the field and dry lot during 1932. *Crotalaria spectabilis* was rejected entirely by 5 different cows allowed access to the pasture during one or another of 5 tours. Three Jersey cows were used in the hay-palatability trials with eight species of *Crotalaria* from October 21 to November 16, 1932. These animals consistently left *C. spectabilis* in the rack as the last choice of the eight species. On November 16 three yearlings from the beef cattle herd replaced the cows in the dry lot, and at that time the latter animals were transferred to a maintenance trial on *C. intermedia* hay. Specific animals and the lengths of the tours on the several trials are outlined in detail in table 2. These summaries are compiled from the observations of cattle made daily by two or more station workers.

Four of the cows were changed from *Crotalaria intermedia* hay to corn silage and Bermuda grass (mixed) pasture on March 2, 1933. Cow no. 352 was autopsied for definite examination in search of any indications of toxicity of the forages eaten. The cows exhibited no symptoms of toxicity, nor were any gross or histological lesions evident in the organs and tissues upon autopsy, which was conducted by Drs. E. F. Thomas and C. F. Ahmann of this station. The feed of three of these cows had been limited strictly to *C. intermedia* from November 17, 1932, to March 1, 1933, inclusive—a period of 105 days, and they had had access to it previously, as noted in table 2.

TABLE 2.—*Duration of feeding trials with cattle, and animals used, during the 1932 grazing season and the winter of 1932–33, the species of Crotalaria described in table 1 being used*

Nature and duration of trial	Feedings [1] given to animal [2] indicated					
	No. 151	No. 228	No. 264	No. 296	No. 350	No. 352
Palatability of green forages:						
June 18 to July 2	[3] ×			[3] ×		
July 28 to Aug. 13			[3] ×		[3] ×	
Aug. 14 to Aug. 27	[3] ×			[3] ×		
Oct. 4 to Oct. 20		[3] ×		[3] ×		
Palatability of dried hays:						
Oct. 21 to Oct. 28	[4] ×	[4] ×		[4] ×		
Oct. 28 to Nov. 2	[4] ×	[4] ×		[4] ×		
Nov. 2 to Nov. 7	[4] ×	[4] ×		[4] ×		
Nov. 7 to Nov. 12	[5] ×	[5] ×		[5] ×		
Nov. 12 to Nov. 16	[4] ×	[4] ×		[4] ×		
Maintenance trial with *C. intermedia:*						
Nov. 17 to Dec. 16	×	×		×		
Dec. 17 to Jan. 17	×	×		×		×
Digestion trials with *C. intermedia:*						
Jan. 18 to Feb. 11	[6] ×	[7] ×		[6] ×		[7] ×
Feb. 12 to Mar. 1	[7] ×	[6] ×		[7] ×		[6] ×

[1] × signifies feedings given on the dates mentioned.
[2] Dates of autopsies: No. 151, May 2; no. 228, Mar. 31; no. 296, Mar. 23; no. 350, Apr. 14; no. 352, Mar. 2.
[3] No *C. spectabilis* eaten.
[4] *C. spectabilis* rated last in relative palatability.
[5] No *C. spectabilis* hay offered.
[6] Naturally cured *C. intermedia* hay.
[7] Artificially cured *C. intermedia* hay.

No chronic symptoms had developed between the termination of the maintenance trials and the dates of autopsies of these animals. Indications of toxicity particularly sought in these cases included loss of appetite, dejection, blood in the feces, and nasal hemorrhage. Gross and histological examinations upon autopsy were made particularly upon the liver, heart, kidneys, mesenteric blood vessels, and the fatty tissues. The autopsies were attended and tissues examined by four or more persons including the authors, Drs. E. F. Thomas, C. F. Ahmann, and others of the experiment station and the United States Department of Agriculture staffs. No symptoms were observed, either acute or chronic, of toxicity of the species of *Crotalaria* eaten in these trials other than *C. spectabilis*.

Three native yearling cattle—2 steers and 1 heifer—were used in continuing the test of the relative palatabilities of artificially cured crotalaria hays. Previous to this time, these animals had received carpet, centipede, and mixed native pasture grasses. The log of these palatability trials is given in table 3.

TABLE 3.—*Relative palatability of 8 species of artificially dried crotalaria hays, as indicated numerically according to order of preference and rate of consumption by 3 yearling cattle in 1932*

Duration of trial	Numerical palatability rating of indicated species of *Crotalaria*							
	Intermedia	*Usaramoensis*	*Incana*	*Grantiana*	*Lanceolata.*	*Anagyroides*	*Striata*	*Spectabilis*
Nov. 16 to Nov. 23	1	2	3	4	5	6	8	7
Nov. 23 to Nov. 29	1	2	3	4	6	7	8	5
Nov. 29 to Dec. 5	1	2	3	4	5	8	7	6
Dec. 6 to Dec. 12	1	2	3	6	4	8	7	5
Dec. 12 to Dec. 19	2	1	3	7	4	8	6	5
Dec. 19 to Dec. 23	1	2	4	6	5	7	8	3
Dec. 23 to Dec. 27	2	1	4	8	5	6	7	3

The last hay-palatability trial terminated on December 27, 1932. The 3 yearling cattle used in the last 7 trials died on January 7, 18, and 20, 1933, respectively. They showed almost total loss of appetite, lack of abdominal fill, no bloat, or hoven, blood in the feces, and two had nasal hemorrhage. Autopsy disclosed hemorrhage from blood vessels in the mesentery, heart, muscle coronary and body fat, and in the mucous layer and submucosa of the trachea; a mottled appearance of kidneys and liver; and hemorrhage in the nasal sinuses of two animals.

Although *Crotalari spectabilis* had been withdrawn from the feeding rack on December 28, 1932, the second yearling steer showed intestinal hemorrhage, as evidenced by bloody feces on January 12, 1933, which increased in severity on the 13th. The two animals surviving at this date became less animate; ate less of the crotalaria hays, and their feces were still darker on January 16. Nasal hemorrhage was apparent in the second steer on January 17. This animal was killed for autopsy on January 18, shortly prior to the approaching natural death. The yearling heifer died on January 20.

The autopsy findings, as summarized by Drs. E. F. Thomas, A. L. Shealy, and C. F. Ahmann, are as follows:

History.—Three cattle had been feeding on crotalaria hay. They showed loss of appetite for several days. Bloody feces, and a tinge of blood from the left nostril (of the second steer) were evident.

All mesenteric fat contained numerous petechial hemorrhages. Anterior portion of the frontal sinus showed ecchymotic areas; blood clots in posterior frontal sinus. A few worms were embedded in wall of esophagus (not identified). Extensive hemorrhages along the mucous membrane and submucosa of the trachea.

Endocarditis; myocarditis, and epicarditis (especially along the interventricular groove). Pericardium showed numerous petechial hemorrhages. Liver finely mottled with red. Gall bladder showed numerous petechial hemorrhages. Spleen probably enlarged slightly. Wall of abomasum showed the mucous membrane edematous. Mucous membrane of the small intestines appeared normal.

Submucosa of small intestine showed ecchymosis and petechial hemorrhage.

Large intestine.—No hemorrhage of mucous membrane, but an abundance of blood clotted in the lumen. Fecal matter was firm; some hard. Urinary bladder showed a hemorrhagic area. The brain was congested, with gelatinous material in the sulci (grooves). Lymph glands showed very little congestion. All body fat was very yellow, and showed petechial hemorrhages throughout.

Tissue from the liver, kidney, and spleen was fixed for examination.

Typical lesions of *Crotalaria spectabilis* poisoning may be seen in the left view of the heart (fig. 1), liver (fig. 2), gall bladder (fig. 3), inner surface of the trachea (fig. 4) and mesentery (fig. 5) of the yearling steer which was killed just prior to natural death. These photographs show the hemorrhages resulting from leakage of blood from the vessel walls. The lesions compare closely with those produced in chickens by Thomas, Neal, and Ahmann (*10*), using *C. spectabilis* seeds, and also the alkaloid extracted from the seeds, leaves, and stems of this plant.

AUTOPSY OF THIRD CASE

The third yearling (heifer) died January 20, 1933, probably about 10 hours prior to autopsy. She had been off feed for 13 days, and had passed considerable blood with the feces during the last 2 days.

The mesenteric fat showed little hemorrhage. Petechial and ecchymotic hemorrhages were present in the submucosa of the intentines. The lungs apparently were normal. There was a severe

hemorrhagic condition of the heart muscle. The liver showed a few hemorrhagic areas. The gall bladder had several ecchymotic areas, and there also were numerous ones in the spleen. The urinary bladder appeared normal. The lumen of the large intestine contained blood and blood clots. The pericardium showed numerous petechial hemorrhages. Amber fluid was present in the pericardial sac. Numerous subcutaneous petechial hemorrhages also were present.

It is to be noted particularly that none of the cows that refused to eat *Crotalaria spectabilis* in the grazing-palatability trials, or in the

FIGURE 1.—Left view of heart, taken from a yearling steer affected with chronic *Crotalaria spectabilis* poisoning, showing petechial hemorrhages in the coronary fat and the outer walls of the auricles and ventricles. The hemorrhagic condition is more general in the ventricular walls adjacent to the left interventricular groove and toward the apex.

trials with artificially cured hays, showed any indications of *Crotalaria* poisoning, either in life, or upon autopsy. However, the three yearlings that changed their order of preference of these species, and consumed 98 pounds of *C. spectabilis* hay between November 16 and December 28, 1932, died of *Crotalaria* poisoning between January 7 and 20, 1933. Since they also had access to other feeds, and since there could still be a question as to *C. spectabilis* specifically, a supply of the artificially dried hay was preserved pending availability of a suitable animal for an exact trial.

SPECIFIC TEST OF CROTALARIA SPECTABILIS

E-53, a dun and white native steer just under 8 months of age and weighing 300 pounds, was supplied from the native herd at the experiment station. This animal had suckled its dam from birth, and had been on mowed-grass pastures during its lifetime. It had not had access to *Crotalaria spectabilis* previously, insofar as is known. The animal was healthy and extremely vigorous.

FIGURE 2.—Liver of a yearling steer affected with chronic *Crotalaria spectabilis* poisoning, showing the surface finely mottled with red as the result of hemorrhage.

Artificially dried *Crotalaria spectabilis* hay prepared in 1932, was offered to this calf, which refused to eat it. The chopped hay was mixed with other feeds, which it still refused to eat. Water was available in the dry lot at all times. A stomach tube was passed, weighed quantities of ground *C. spectabilis* hay were suspended in

water, and the animal drenched therewith. The record of these feedings of *C. spectabilis* hay was as follows:

1933	*Pounds*
Dec. 12	0. 5
Dec. 13	4. 0
Dec. 14	3. 5
Dec. 15	1. 5
Total	9. 5

Blood was present in the feces the morning of December 14 and the animal died about 4:30 p. m. the next day.

FIGURE 3.—Gall bladder from a yearling steer affected with chronic *Crotalaria spectablis* poisoning, showing numerous petechial hemorrhages. The bile appeared normal in color and consistency.

E–53 was photographed a few minutes prior to death (fig. 6). Death occurred quietly, the animal lying on its side, the only struggle being a slight paddling of the feet. The autopsy was made on the warm body before the onset of rigor mortis.

The blood had not clotted, nor did it clot readily upon the severing of the jugular vein. The subcutaneous blood vessels, those in the adipose tissue and along the small intestines, were enlarged. The thymus and spleen appeared normal. A slight amount of straw-

colored fluid had accumulated in the peritoneal cavity. Urine in the partly filled bladder was of a normal pale-amber yellow color.

Petechial hemorrhages were present in the mesentery, on the caecum, and in the gall-bladder wall. Mesenteric lymph glands were enlarged. The lumen of the lower small intestine, all of the large intestines, and rectum contained the bloody material mentioned previously as appearing in the feces. No hemorrhages were apparent on the stomach compartments nor in the lungs. The kidneys were

FIGURE 4.—Inner surface of the trachea of a yearling steer affected with chronic *Crotalaria spectabilis* poisoning, showing extensive hemorrhages along the mucous membrane and submucosa of the trachea. The trachea was opened along the ventral surface.

darker, with a slight turkey-egg mottling. The liver also was a dark red-blue color, and quite friable. The gall bladder wall was thickened and spongy in texture. The bile appeared normal in color and consistency.

The heart showed petechial hemorrhages in the coronary fat, on the auricles and along the interventricular groove. Parts of the inner wall of the heart, the papillary muscles and some of the chordae tendineae showed marked hemorrhages, as seen in figure 7.

The case of E–53 is considered acute, whereas those of the three animals dying previously were chronic.

FIGURE 5.—Mesentery and intestines of a yearling steer affected with chronic *Crotalaria spectabilis* poisoning, showing numerous petechial hemorrhages in the mesenteric fat, on the outer surface of the small intestines and the apex of the caecum. The submucosa of the small intestines showed ecchymoses and petechial hemorrhages. There was an accumulation of clotted blood within the lumen of the large intestines.

FIGURE 6.—An 8-month-old steer photographed less than half an hour before dying from acute *Crotalaria spectabilis* poisoning. Loss of appetite and lack of animation were evident in the acute and chronic cases alike. Dried blood from a nasal hemorrhage is visible below the right nostril. The hair is harsh and unkempt.

TRIALS WITH SILAGE

In 1932 four species of *Crotalaria*, including *C. spectabilis*, were ensiled in 1-ton laboratory silos (7) for use in comparing the relative palatability of these species. *C. intermedia* was first choice of the

cattle, and was eaten liberally. The *C. spectabilis* ranked third in order of preference by the cattle. The latter silage had a dark color, and animals eating it voided dark-colored feces. These silages were offered to 30 cattle over a 16-day period. No irregularities were observed with these cattle other than the temporary discoloration of the feces.

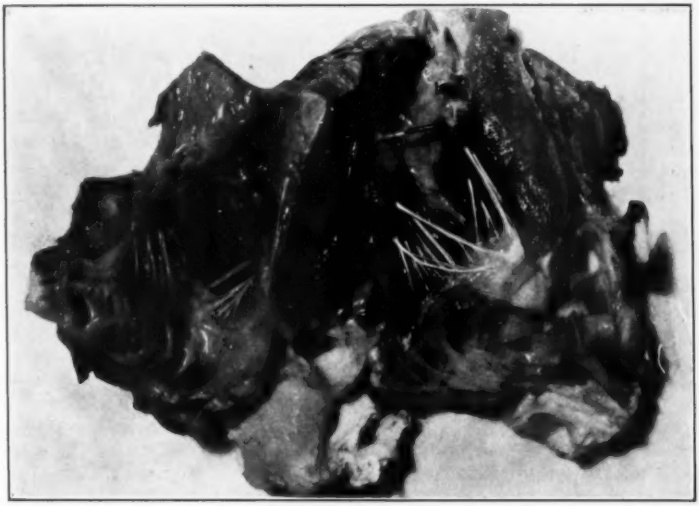

FIGURE 7.—Inner walls of the heart of a 300-pound steer that died of acute *Crotalaria spectabilis* poisoning. There are extensive hemorrhagic areas on the inner lining of the ventricles, the hemorrhages extending deeply into the heart muscle. The papillary muscles and a few of the chordae tendineae also show hemorrhages.

Two palatability studies conducted in 1933 dealt with six species of *Crotalaria* as silage. These were of 11 days' duration each, and involved 51 cattle. As noted in table 1, no *C. spectabilis* was offered at this time, nor were indications of toxicity observed in the cattle during, or after, eating these species of *Crotalaria* as silage.

Twenty-five tons of *Crotalaria intermedia* were ensiled in a commercial silo, and fed to dairy cows in a 90 days' double-reversal feeding trial, and to four steers over periods of 59 and 31½ days in a study of coefficients of digestibility. No indications of either acute or chronic toxic influences were observed in any of the cattle used in investigations on the *C. intermedia* silage.

SUMMARY

Grazing and feeding trials indicate that at least 8 out of 10 introduced species of *Crotalaria* are probably not toxic to cattle. *C. retusa* was not grazed. *C. spectabilis* is definitely toxic to cattle.

One acute and three chronic cases of *Crotalaria spectabilis* poisoning in cattle are discussed, with symptoms and lesions as noted. *C. spectabilis* Roth is added to the list of species of this genus definitely proved toxic to domestic animals.

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CLASSIFICATION OF CHICK-EMBRYO POSITIONS AT DIFFERENT AGES AND MALPOSITION AS A CAUSE OF MORTALITY [1]

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INTRODUCTION

Growth of the chicken embryo is accompanied by gradual changes in position in accordance with the necessity of breaking through the shell and shell membranes at hatching time. Failure to secure a correct position with the beak near the air cell, the egg tooth directed toward the shell, the pipping muscle free to function, and the feet in a position for body rotation, may either hinder or prevent escape. As a consequence the ineffective position would appear to constitute a mechanical lethal factor, and malposition would be considered a cause of death.

A direct causal relationship between malposition and embryonic mortality has been considered by Sanctuary (*6*),[2] Hutt (*3*), Hutt and Cavers (*4*), and others. Hutt and Pilkey (*5*) have observed malpositions in more than 50 percent of the "dead in shell" embryos. They state that malposition is "largely responsible for the peak of mortality occurring during the last 3 days of incubation."

Nevertheless, the causal relationship that is supposed to exist between position and death has been defined only in part. Byerly and Olsen (*1, 2*) have shown that both gravity and air hunger effect changes in some of the positions of the embryo. Hutt and Pilkey (*5*) consider position of the egg during incubation and orientation of the primitive streak important factors in effecting malposition. But these malconditions are not commonly met when proper methods of incubation are followed. Position I as indicated by Sanctuary (*6*), with the head between the legs, may be produced artificially by inverted incubation through the effects of gravity and of air hunger. That does not furnish an explanation for the cause of its occurrence under nonexperimental conditions with proper methods of incubation applied.

Furthermore, all six malpositions so far reported (table 1) have been recorded from the dead-in-shell. A classification of the various positions taken by normal embryos, that is, the positions that do not interfere with hatching, has never been recorded, as far as the writer is aware. Yet, before a causal relationship can be considered as existing between embryo position and embryo mortality, i. e., before a position can be termed malposition in the sense that it hinders or precludes hatching, a knowledge of the relative occurrence of positions taken by normal chicks is essential.

[1] Received for publication Feb. 14, 1935; issued July 1935.

[2] Reference is made by number (italic) to Literature Cited, p. 931.

TABLE 1.—*Malpositions of chick embryos* [1]

Position no.—	Described by—	Description
I	Sanctuary, 1925	Head between the thighs.
II	do	Head in small end.
	Reaumur, 1751	
III	Sanctuary, 1925	Head to left instead of under right wing.
IV	Hutt, 1929	Head normal, but embryo rotated so that the beak is buried away from the air cell.
V	Smith, 1930	Feet over head.
VI	Hutt and Cavers, 1931	Beak over right wing or over and under wing.

[1] Hutt and Pilkey (*5, p. 4*).

METHODS AND PROCEDURE

In connection with a study of the causes of embryonic death instigated by the writer at the Maine Agricultural Experiment Station in 1927, a systematic mortality code has been developed to record teratas and defects of embryos or of embryonic tissues which might in any way give a clue to the causes of death. At the same time, as supplementary information on the cause of death, data have been accumulated on the positions of the embryos.

Early in the work it became evident that malposition is not in itself always a cause of death, (1) because position was found to vary with age (the position of a 15-day-old embryo retained by an embryo 21 days old indicated, among other possibilities, a retarded development), and (2) because there appeared a large number (upward to 12 percent) of obviously defective embryos. These defective embryos were in characteristic abnormal positions, but their failure to hatch was a result of abnormal development and not of malposition. In fact, defective embryos occurring most frequently (termed A3) were characterized by nanism and torticollis with the head twisted sideways or even backwards. The failure to emerge was not the result of an awkward position, since the embryos usually had failed to assimilate all of the albumen, were lethargic, and lacked directive movement. Granted that their positions were not advantageous to emergence, it would have been impossible in any case for such individuals to hatch.

If the position of the embryo is to be used as a means of determining the causes of failure to hatch, it is necessary to determine first the cause of each position type, i. e., whether or not it actually prevents the act of pipping the shell or is an earmark of a more deeply-seated physiological defect.

As an approach to this phase of the subject a classification was made of the orderly changes in position of embryos with age. The purpose was threefold: (1) To obtain a position type for each age, (2) to obtain information on the variability of positions so that malposition may be more clearly defined, and (3) to observe the process and possible causes for the different position types. This paper indicates the order of changes in position of embryo from the fifteenth day of incubation to the twenty-first, and presents a systematic chart of those changes together with the variations in position that an embryo may take whether normal or abnormal. The chart links all six positions previously recorded by other workers with the new positions found by the writer and includes a prediction of positions to be found by following through the mirror image of the positions so far discovered.

SYSTEMATIC CLASSIFICATION OF POSITION TYPES

A systematic classification of embryo position which covers all possibilities is sometimes difficult to accomplish, since unsuspected position types appear in new sources of material. Consequently a flexible classification was made that could be expanded if necessary.

The code is based upon a division of positions into independent events, the occurrence of any one of which does not occlude the occurrence of any other, and the subdivision of each independent event into mutually exclusive events, only one of which may occur in any embryo.

The four independent groupings are as follows:

A. Position of the air cell in respect to the egg.
B. Orientation of the embryo as a whole within the egg.
C. Position of the head, beak, and neck in relation to the body.
D. Position of the beak in relation to the air cell.

The subdivisions of each independent group into mutually exclusive events or positions are listed in table 2.

From the foregoing classification it is possible to describe with exactitude the position of any embryo from 15 to 21 days of age. Thus the normally orientated embryo is described as 1–a–6–a:

1. Air cell in base of egg (large end).
a. Embryo with head in large end of egg.
6. Head turned to right side, with beak under right wing.
a. Beak near air cell.

There are a few unclassified conditions that may interfere with hatching. These are listed under abnormal feet positions and include the following:

1. Agrippa, feet first.
2. Legs crossed.
3. Feet tied by membranes or by the yolk stalk.
4. Membranes tied about the body or neck.
5. Hernia due to kicking.
6. Broken yolk sac due to kicking.

The foregoing accidental defects have been placed under a separate classification.

TABLE 2.—*Code for the classification of chick-embryo positions, including a code for the orientation of the embryo in respect to the egg axis and the air cell, based on four independent events*

A, air cell	B, body position	C, position of head and beak, and direction of neck twist			D, proximity of beak to air cell
		Head	Beak	Primary twist in neck	
1, in base. 2, in tip. 3, at side.	a, head in base. b, head in tip. c, head and feet in tip. d, head and feet in base.	0, between legs, feet over head	Straight down	None.	a, beak near air cell. b, beak away from air cell (1 inch or more).
		1, to center between legs	do	None.	
		2, to center between legs	Tip to right.		
		3, to center between legs	Tip to left.		
		4, across breast	Points to right	Sinistral.	
		5, across breast	Points to left	Dextral.	
		6, to right side	To right, tip under right wing	Sinistral.	
		7, to left side	To left, tip under left wing	Dextral.	
		8, to right side	To right, tip under and over right wing.	Sinistral.	
		9, to left side	To left, tip under and over left wing.	Dextral.	
		10, to right side	To right, tip over right wing	Sinistral.	
		11, to left side	To left, tip over left wing	Dextral.	
		12, on right side	To front and left	Do.	
		13, on left side	To front and right	Sinistral.	
		14, on right side	To back or above	Right torticollis.	
		15, on left side	To back and above	Left torticollis.	
		16, to front	To front and above	S-curve forward and upward.	
		17, to back (above and over)	To back (above and over)	Retrocollis.	

NORMAL CHANGE IN POSITION OF THE EMBRYO WITH AGE

During the season of 1931 observations were made on the normal change in position taken by the embryo as it advances in age from a small floating primitive streak to a hatching chick tightly packed in the shell. Positions were classified according to the scheme presented in table 2. For the effect of age, consideration was given only to head, wing, and neck positions with all other factors constant. The position frequencies for each age, from 15 to 21 days of age, are shown in table 3.

TABLE 3.—*Changes in head position of chick embryos with incubation age and frequency of the various head positions*

Incubation age (days)	Head between legs, 0-1-2-3 [1]	Head across breast, 4-5 [1]	Wry neck, 12-17 [1]	Normal, 6-8-10 [1]
15	[2] 17	3		
16	[2] 50	15	4	
17	83	[2] 106	13	
18	33	[2] 58	9	4
19	4	2	2	[2] 12
20	4			[2] 14

[1] Code numbers refer to the embryo positions described in column C in table 2.
[2] = modal class.

The results show that head positions 1, 2, and 3 predominate in embryos from 15 to 16 days of age, that head positions 4 and 5 predominate in embryos from 17 to 18 days of age, and that head positions 6, 8, and 10 are the most common for embryos 19 days old and over.

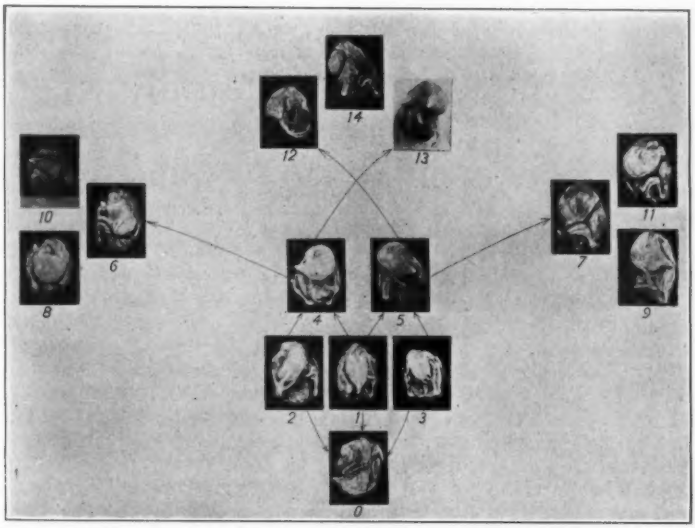

FIGURE 1.—Codification of head positions of 21-day-old chick embryos: 0, Extreme form of 1, 2, 3; 1, 2, 3, similar to 15- and 16-day-old; 4, 5, similar to 17- and 18-day-old; 6, typical hatching position; 8, 10, variations of position 6; 7, 9, 11, reverse of positions 6, 8, 10; 12, 13, 14, defective.

In other words, the head position is at first (at 15 days of age) straight down between the thighs nearly to the tail. The tipping of the beak to the right or to the left apparently indicates the direction the beak will take even before the head is raised to the side of the body. At 17 days the head is drawn up over the breast where it turns either to the right or to the left according to its normal tendency. At 18 days the head lies across the breast.

TABLE 4.—*Frequency distribution of embryo positions*

Code [1]	Hatchable [2] alive, 1931	Pipped, dead, 1931–32	21 days old, dead, 1931–32	18 to 20 days old, dead, 1931	20+21+pipped dead, 1932		Abnormal 21 days old, 1931–32
					Male	Female	
A, air cell:							
1	218	339	942	168	375	423	157
2		1	2		1		
3		1	9	1	4	6	1
?		3		1			
B, body position:							
a	212	335	891	151	342	393	141
b	6	7	44	15	29	33	8
c		1	14	3	9	2	4
d			4	1		1	4
?		1					
C, head position:							
0			1		1		
1			18	7	4	10	4
2		2	60	12	14	17	7
3			72	14	18	13	5
4		9	79	16	34	37	42
5		1	40	7	10	15	4
6	125	217	218	53	143	144	11
7			104	12	31	33	4
8	27	63	27	1	23	29	13
9			2			3	
10	66	51	192	32	64	75	5
11			4			1	3
12			111	13	25	37	41
13		1	22	3	17	4	16
14			1				1
15			1				
16							
17			1		1		1
D, beak to air cell:							
a	212	428	518	96	230	263	44
b	6	30	444	73	150	166	110

[1] Coded according to description in table 2 under columns A, B, C, D.
[2] From examination of chicks as they hatched. A few might have died as "pipped."

Head positions 4 and 5 of the 18-day-old embryos are in critical stages, since they are the axes about which and on which the final position of the hatching chick depends. An embryo at 18 days of age with its head across the breast (4) with tip of beak pointed to the right has an alternative of five different positions on the nineteenth day, i. e., either to slip backwards to the left and form the position 13 or 15 (the wry-neck class) or to slip to the right and form the so-termed "normal position" (6), with beak protruding under the right wing, or one of the variants (8) or (10).

Should the beak be pointed toward the left, the opposite directions would be taken with five different positions among the left possibilities (mirror images of the right sides). In some wry-neck embryos (torticollis) the head continues to move backward along either side (positions 14 and 15). Another class of wry-neck embryos (retrocollis) appears with the head thrown straight back (positions 16 and 17). Torticollis also appears in embryos 14 days old or younger, apparently without going through this serial change in position.

Table 2 is made to conform with the logical changes in head position taken by the embryo as it develops from the fifteenth day. In figure 1 are shown all of the head positions found in 21-day-old embryos. Attention must be called to the fact that among these mature embryos will be found head positions 1, 2, and 3, which are typical for the 15- and 16-day-old embryos. As 21-day-old embryos they are in retarded positions. In the same way head positions 4 and 5 indicate a partial retardation since they are typical for embryos of 17 and 18 days of age. Head positions 12, 13, and 14, on the other hand, are the positions taken by defective embryos. Head positions 15, 16, and 17 have been found in defective embryos which died before the twentieth day of incubation. In table 4 the frequency distribution of embryo position is listed for each independent event, and again for each mutually exclusive head position as taken by various types of embryos: By normal hatchable chicks, by those that pipped and died in the shell, by those that died at from 18 to 21 days of age, in certain cases by males contrasted with females, and by those abnormal at 21 days of age.

POSITION OF ABNORMAL EMBRYOS

Abnormal embryos, like the A3 previously mentioned, together with extreme examples of chondrodrystrophic embryos, are listed as to position under the term abnormal in table 4. As a group they do not differ significantly from other types of embryos in regard to air cell or body position. But in head position they are most frequently classified as partially retarded (head position 4) or as wry-neck (head positions 12 and 13). They also have a general disregard for the air cell since in over 70 percent of the cases the beak is not near the air cell. This figure is in distinct contrast to the percentage of hatchable chicks and pipped chicks which failed to find the air cell, i. e., 3 and 7 percent respectively.

DETERMINATION OF THE CAUSE OF FAILURE TO HATCH FROM THE POSITION OF THE EMBRYO

Contrary to the statements cited that "malposition is one of the primary causes of embryo mortality" the results of this analysis show that a large portion of the embryos that fail to hatch are in positions which can be considered as the secondary effect of some more fundamental defect. The correlation assumed to exist between these positions and mortality is therefore, at times, spurious. As an example of the fallacy post hoc ergo propter hoc, it may be likened to the inference that since rabbits poisoned in their burrows by strychnine are found in strained and peculiar positions, they must have failed to escape from their warren and from death because of these eclamptic positions. In a similar manner the fundamental causes of the defective condition in an embryo, whether they be from the effects of inbreeding, of faulty nutrition, or of careless incubation methods, will interfere with the normal sequence of body shiftings and as a consequence, will leave the embryo in a retarded or strained position when death occurs.

Malposition I of Sanctuary (*6*), which is coded here as head positions 1, 2, and 3, together with the extreme form, malposition V of Smith (*7*), (coded here as head position O) may now be used to indicate

three possible causes of death: (1) Air hunger when the air cell is misplaced, (2) gravity when the egg is incubated upside down, and (3) factors delaying development previous to the fifteenth day of incubation. Likewise head positions 4 and 5 appear to be due to the same causes to a lesser degree.

The third cause of death, i. e., that which manifests itself by delayed development, has not heretofore been considered a factor in causing malposition. It is obvious from table 3 and table 4 that delayed development in whatever form it may occur is a phenomenon associated with embryonic mortality.

Attempts to bring about a normal position in such embryos are wasted. A slight degree of debility will cause an embryo to vacillate in taking a normal position regardless of the artificial attempts to turn the egg and force it into a more appropriate orientation. Attempts to force such malposed embryos by egg turning will result only in another type of malposition.

Thus when Hutt and Pilkey (*5*) found that tilting the eggs during incubation produced an increase of 2.10 percent in positions I, III, and VI, they found that horizontal incubation resulted in an increase of 2.73 percent in positions II and IV. Their attempts to combine the advantages of both methods of turning should prove futile if we look upon these malpositions as a form of general debility. In such a case the correcting factor might be found in feeding to increase vigor of the embryo rather than in methods of turning the eggs during incubation. These slightly retarded embryos, together with the obviously defective embryos, constitute a large portion of the dead in shell. Their positions may suggest some of the causes for death, but the positions do not as such form barriers for their emergence.

Malposition VI of Hutt and Cavers (*4*), which is coded here as head positions 8 and 10, occurs in 21-day-old embryos failing to hatch in approximately 42 percent of the cases. But the same positions are found to occur among hatchable chicks with the same frequency, i. e., 42 percent (table 4). Consequently these 2 head positions appear to be variations of the more typical normal head position 6 and should therefore not be termed malpositions.

SUMMARY AND CONCLUSIONS

A systematic classification of the positions taken by normal hatchable chick embryos and by the dead-in-shell shows that position as such is not always, and may only occasionally be, the real cause for failure to hatch. Position VI of Hutt and Cavers (*4*) has been found to occur in hatchable chicks with a frequency equal to that found in dead-in-shell chicks; it cannot therefore be termed a malposition. Position III of Sanctuary (*6*) and position V of Smith (*7*) have been shown to be due to a delayed development taking effect prior to the fifteenth day of incubation. Defective embryos in new positions have been associated with nutrition of the dam. Thus the correlation between position and embryo mortality is in part spurious. Postdiction of causes for failure to hatch from position of the embryo become apparent only after the conditions are resolved into more fundamental causes, i. e., effects of breeding, of feeding, and of incubation methods, many of which bring about a retardation in the normal shift in position as a secondary after-effect of the true lethal factor.

A position code is presented for the classification of embryos. It is based upon a combination of independent and mutually exclusive events so that the position of any embryo may be easily described by means of a four-letter code.

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